Extracting and purifying isoflavones from defatted soybean flakes using superheated water at elevated pressures

Chang Li-Hsun, Cheng Ya-Chuan, Chang Chieh-Ming*

Department of Chemical Engineering, National Chung Hsing University, Taichung, 402 Taiwan, ROC

Abstract

This paper focusses mainly on improving superheated water extraction conditions using the simulation optimization approach known as "steepest ascent". The study examines the effect of operating conditions on the amount of five isoflavones extracted from defatted soybean flakes (DSF) using superheated water extraction (SWE), stirred solvent extraction, Soxhlet methanol extraction, and pressurized water extraction (PWE). Amberlite XAD16-HP adsorbent was used to purify five isoflavones obtained by reverse phase solid extraction. HPLC analysis was conducted to quantify these five isoflavones as proportions of the amount of total isoflavones (TIF). The steepest ascent design for SWE revealed that the optimum condition was 110 °C and 641 psig (4520 kPa) over 2.3 h of extraction. Under these condition, 3937 TIF μg/g $_{DSF}$ was produced. Solid phase purification, by Amberlite XAD16-HP absorption, yielded a solid with 37.0 purity and 49.0 de-sorption recovery of total isoflavones.
© 2003 Elsevier Ltd. All rights reserved.

Keywords: Superheated water; Defatted soybean flakes; Isoflavones; Extraction and purification

1. Introduction

Soybean products are popular among Asians and vegetarians. Isoflavones, such as glycosides daidzin (D), glycitin (Gly), and genistin (G), are quite abundant in soybeans (Chiang, Shih, & Chu, 2001). Aglycones such as daidzein (De) and genistein (Ge), derived from these glycosides, are frequently examined in their chemical structures. These isoflavones have several biological effects and are considered to be anti-carcinogenic (Nguyenle, Wang, & Cheung, 1995), beneficial to human health and present in several soybean foods in concentrations from 1000 to 5000 μg/g (Coward, Barnes, Setchell, & Barnes, 1993). Daidzin was especially found to be present at high concentrations in hypocotyls of soybeans (Tani, Katsuki, Kubo, Arichi, & Kitagawa, 1985). Extraction of isoflavones from soybeans using organic solvents is difficult. Some conventional approaches employ very efficient room temperature- or cold 80% methanol extraction (Wang & Murphy, 1994). Murphy et al. (1999), Murphy,

Barua, and Hauck (2002), and Nurmi, Mazur, Herionen, Kokkonen, and Adlercreutz (2002) compared methanol, ethanol, acetone and acetonitrile (AC+ACN) for extracting isoflavones from various soy food matrices and demonstrated that AC+ACN appears to be the best solvent, regarding the yield and the recovery of total isoflavones. But for soy flour, Murphy et al. (1999, 2002) showed that MeOH was as efficient as AC+ACN for extracting isoflavones and was the best solvent for extracting aglycones. However, these organic solvents leave a toxic solvent residue in the extract when these extraction methods are used.

The "Green High Pressure Process" is a recently developed technology used to produce natural foods. This process does not change covalent cross-linking and a high pressure has been found to be able to improve texture and the quality of the food (An-Erl King, Chang, & Kwo, 1994). Chandra and Nair (1996) detailed supercritical carbon dioxide extractions of isoflavones, including daidzein and genistein in soybean products. Rostagno, Araujo, and Sandi (2002) recently reported 40.5% recovery of isoflavones (daidzein, genistein and genistin) by using SC–CO_2, which is much lower than that by Soxhlet 80% methanol extraction

---

* Corresponding author.
E-mail address: cmchang@dragon.nchu.edu.tw (C. Chieh-Ming).

($R = 100\%$). However, supercritical fluid is not feasible for extracting aglycones, such as daidzein and genistein, because isoflavones, as aglycones, are relatively scarce while glycosides are relatively abundant in soybeans.

Liquid water under enough pressure to maintain the liquid state, at above 100 °C but below its critical temperature of 374 °C, is referred to as superheated water (SW) or subcritical water (Basile, Carmona, & Clifford, 1998). Water below its boiling point is referred to as pressurized water (PW). However, heavy hydrocarbons (M.W. > 250) are generally believed to be appreciably soluble in water at temperatures near or below the critical temperature, according to measurements made in steel autoclaves (Sanders, 1984). The recoveries of all target components achieved by subcritical water extraction compare favourably (90–120%) with those achieved by conventional solvent extractions (Yang, Belghazi, Lagadec, Miller, & Hawhorne, 1998). This work examines the use of a high-pressure hot water process to extract five isoflavones from defatted soybean flakes (DSF). An experimental design was adopted to optimize extraction conditions, and five isoflavones were purified by solid-phase column absorption (Huang & Hsieh, 1998). Furthermore, reversed phase high-performance liquid chromatography (RP-HPLC), with UV detection, was also used to quantify five isoflavones (TIF) extracted and purified from defatted soybean flakes.

2. Materials and methods

2.1. Reagents and materials

DSFs were obtained from the Central Union Oil Corp., Taiwan, and were stored at 5 °C before use. Deionized water was prepared using a Milli-Q system. Methanol (MeOH), acetonitrile, acetone, trifluoroacetic acid (TFA) and ethyl acetate (EA) were obtained from Merck (Germany). Standard isoflavones D (>98%, Fluka, Switzerland), Gly (ICN Biomedicals, USA), G (>97.1%, Sigma, USA), De (>98%, Research Biochemicals International, USA) and Ge (>98%, Sigma, USA) were used to prepare a standard mixture for quantitative analysis. All solutions were filtered through 0.45 μm membranes before HPLC analysis.

2.2. Experimental design

Organic solvent (200 ml) was used to extract 20 g DSF for both Soxhlet and stirred extractions. Deionized water (1800 ml) were used to extract 180 g DSF in a 2l agitated autoclave by pressurized water extraction (PWE). The steepest ascent design is a simple linear regression tool (Experimental Design, 2000) and was used to examine the effect of each independent variable on the amount of TIF extracted. Factors (X_i) considered

likely to impact the total concentration of isoflavones (Y, μg/g$_{DSF}$) were extractive temperature, pressure and duration. A three-level, three-factor fractional design was used to establish a central composite design by considering major factors for optimizing extraction conditions and detecting the total amounts of daidzin, glycitin, genistin, daidzein and genistein in the extracts. An optimal point, approaching randomness by being equally predictable in all directions from the centre, was obtained. The levels of the factors presented in Table 1 are shown as coded and the actual levels of those three independent variables. According to Eq. (1), X_1 (°C) is the temperature; X_2 (psig) is the pressure, and X_3 (h) is the time. Thus, the unknown function was assumed to be approximately a first-order response model.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + \varepsilon \tag{1}$$

where b_0 (centre point of system) and b_i (coefficient of linear effects) are constants in the model. The term ϵ is the error of the model. In the calculation, natural variables were transformed into coded variables.

2.3. HPLC analyses

The HPLC analysis of five isoflavones was performed on an Inertsil RP-C8 (250*4.6mm) reverse-phase column with a Hitachi L-4200H UV–vis detector, a Hitachi L-7100 pump and a D-7000 Interface. The column temperature was maintained at 40 °C using a PID thermostat. UV detection was performed at a wavelength of 254 nm and the injection volume was 20 μl. The mobile phase used for analysis was solvent A: 0.1%TFA solution and solvent B: acetonitrile. A flow rate of 1.5ml/min under the initial condition of 88:12 (A : B) was held for 25 min, brought to 22% B in 1 min at a flow rate of 1.0 ml/min, to 25% B in 14 min, to 50% B in 10 min, and to 80% B in 5 min all with a linear gradient. Finally, 80% B was used for 60 min to wash the column.

D, Gly, G, De and Ge (by HPLC chromatography) were separated in sequence with the above gradient elution. Fig. 1 shows HPLC chromatograms of the standard mixture and the SWE extracts. The dominant isoflavones in the standard mixture were D (7.89 μg/ml), Gly (4.02 μg/ml), G (14.4 μg/ml), De (2.62 μg/ml) and Ge (4.02 μg/ml). The R^2 of each calibration curve reached 0.999.

Table 1
Coded and actual levels of three variables

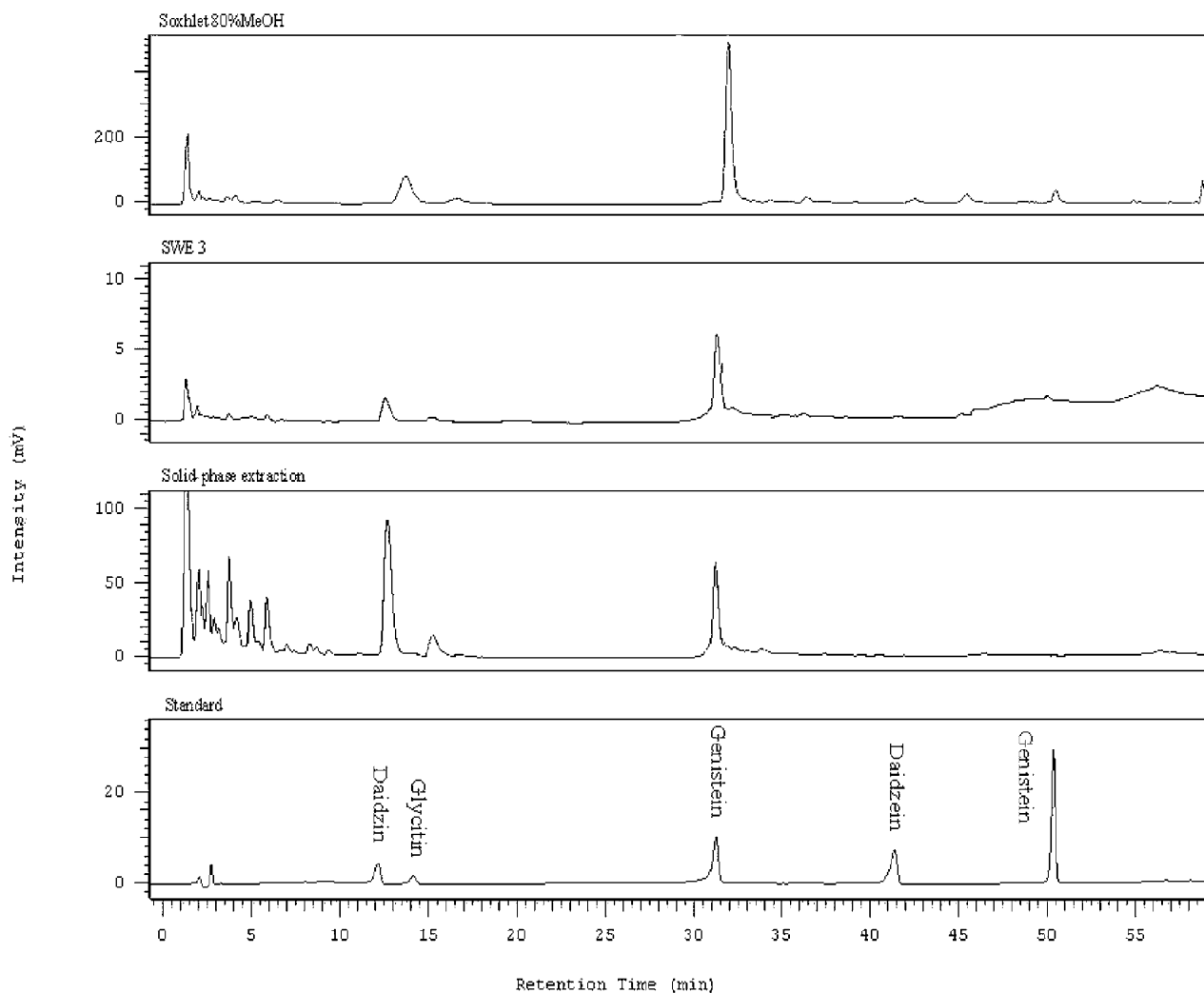| Variable	Coded level of variable		
	−1	0	1
Extraction temperature (°C)	60	80	100
Extraction pressure (psig, kPa)	300, 2169	500, 3548	700, 4926
Extraction time (h)	1	2	3

Fig.1. HPLC chromatograms of typical samples (SWE 3 was diluted by a factor of 50).

2.4. Purifying isoflavones

Partitioning, using solid phase extraction (SPE), is a selective method for completely purifying isoflavones. The adsorbent resin of Amberlite XAD16-HP is a polymeric material supplied as insoluble white beads. It is a nonionic, hydrophobic, cross-linked polymer, with a high surface area and a surface that exhibits aromatic properties, able to adsorb hydrophobic molecules from polar solvents and volatile organic compounds from vapor streams, used here to purify five isoflavones. SPE was performed in batch in a packed bed. The preliminary operations are commonly referred to as a "resin conditioning". Deionized water was initially used to wash the sample and 80% methanol was used to elute it. The flow rates for loading, washing and elution were 1–2 bed volumes per hour. The second purification is performed by liquid-liquid extraction, using ethyl acetate (EA) to extract isoflavones directly from SWE extracts filtered through cellulose paper (Whatman® No. 4).

3. Results and discussion

3.1. General

Soxhlet (80% methano)l extraction was the reference run at the boiling point of the solvent for 12 h. The obtained results indicate that the total isoflavone (TIF) content in the reference run (3948 TIF µg/g DSF) was 1.0–1.5 times higher than that in each extract from PWE. Table 2 presents the amount of TIF expressed as a mean value. Table 3 presents the operating conditions of PWE experiments that produced TIF from 1729 to 3697 TIF µg/g DSF. Compared with Soxhlet (80% methanol) extraction, SWE and PWE did extract more compounds other than TIF ($\beta \times 0.39$–0.65).

3.2. Statistical analysis

Table 3 also shows experimental design data, the total extracted amount (µg/g $_{DSF}$), the yield (Y), recovery (R), concentration factor (β) and separation factor (α_A).

Table 2
Experimental results concerning conventional solvent extractions

Run	Method, solvent	t (°C)	t (h)	TIF[a] (μg/g $_{DSF}$)	Y (%)	R (%)	β	α_A
R1($n=2$)	Soxhlet, 80%MeOH	73	12	3948±174	20.1	100	1.00($\beta_{R1}=4.992$)	1.00($\alpha_{R1}=0.021$)
R2 ($n=2$)	Stirred, 80%MeOH[a]	73	12	3602±6	7.88	91.2		
R3	Stirred, 50%MeOH	80	4	2492	18.4	63.1	0.687	0.643
R4	Stirred, H$_2$O	101	4	1565	17.7	39.6	0.448	0.417

[a] Experiments were performed in triplicate, each time for 4 h. $Y = W_{extract}/W_{feed}$, $R = (W_{TIF})_{extract}/(W_{TIF})_{feed}$, $\beta = (R/Y_{extract})/(R/Y_{feed})$. $\alpha_A = (W_{TIF}/W_{others})_{extract}/(W_{TIF}/W_{others})_{feed}$, $TIF = D + Gly + G + De + Ge$.

When extracted at 100 °C and 700 psig (4926 kPa) for 3 h, the recovery of TIF is close ($R=93.64\%$) to that of Soxhlet 80% methanol extraction ($R=100\%$). However, this PWE shows that $\beta^*=0.613$ is less than $\beta^{R1}=1.00$. Consequently, this extraction is not selective PWE extraction. Table 4 presents the regression coefficients (b_i) and their significance. The results indicate that temperature has a greater effect than pressure and then time, in recovering TIF from DSF. These effects are supported by the analysis of variance (ANOVA) and an F-test determined a linear regression ($F=17.8651 > 9.7795 = F_{(o.o1, 3, 6)}$). Table 5 presents the results of superheated water extractions (SWE) for the simulation-optimization technique known as steepest ascent. Fig. 2 compares the results of conventional and all steepest ascent experiments. Fig. 3 plots the static dielectric constant of water under PWE and SWE conditions. Temperature and pressure are independent operating variables that influence the static dielectric constant of water. The results reveal that the ascent step in the steepest ascent design might reduce the water dielectric constant and the optimum conditions for SWE are extraction at 110 °C and 641psig (4520 kPa) for 2.3 h. The static dielectric constant of water is 53.2. (The water's dielectric constant in the normal state is 80.0.)

Reducing the dielectric constant of water indeed enhanced the amount of TIF extracted from defatted

Table 3
Experimental design based on central composite results of PWE (β^* and α_A^* are normalized by β_{R1} and α_{R1})

Coded level variable[a]								
T (°C)	P (psig, kPa)	t (h)	TIF[b](μg/g $_{DSF}$)	Y (%)	R (%)	β^*	α_A^*	
−1(60)	−1(300, 2169)	−1(2)	1729	19.9	43.8	0.440	0.436	
1(100)	−1(300, 2169)	−1(2)	2700	28.7	68.4	0.478	0.473	
−1(60)	1(700, 4926)	−1(2)	2305	18.0	58.4	0.649	0.645	
1(100)	1(700, 4926)	−1(2)	2918	23.6	73.9	0.628	0.624	
−1(60)	−1(300, 2169)	1(4)	1821	24.3	46.1	0.381	0.377	
1(100)	−1(300, 2169)	1(4)	2838	30.1	71.9	0.479	0.475	
−1(60)	1(700, 4926)	1(4)	2134	24.8	54.0	0.436	0.432	
1(100)	1(700, 4926)	1(4)	3697	30.6	93.6	0.613	0.609	
0(80)	0(500, 3548)	0(3)	2007	26.2	50.8	0.389	0.385	
0(80)	0(500, 3548)	0(3)	1999	26.1	50.6	0.388	0.384	

[a] Experimental runs performed in random order.
[b] TIF, total isoflavones.

Table 4
First order regression coefficient to determine total amount of isoflavones in SWE (* $P<0.05$; ** $P<0.01$)

Effect	Regression coefficient	T test
Intercept	$b_0 = 2003$	12.532**
$X_1 = T$	$b_1 = 520.5$	6.513*
$X_2 = P$	$b_2 = 245.75$	3.075
$X_3 = t$	$b_3 = 104.75$	1.311

Table 5
Steepest ascent design to determine total amount of isoflavones in SWE (β^{**} and α_A^{**} are normalized by β_{R1} andα_{R1})

	X_1 T(°C)	X_2 P(psig)	X_3 T(h)	TIF(μg/g $_{DSF}$)	Y (%)	R (%)	β^{**}	α_A^{**}
(1) Starting point	80	500	2					
(2) Unit level range	20	200	1					
(3) Factors of ascent ratio	520.5	245.75	104.75					
(4) Ascent distance unit [(2)×(3)]	10410	49150	104.75					
(5) Actual ascent distance [(2)×(3)×q[a]]	10	47	0.1					
(6) Steepest ascent experiments								
Starting point:SWE 1.	80	500 (3548 kPa)	2.0	1999	26.1	50.6	0.388	0.384
SWE 2.	100	594 (4196 kPa)	2.2	2941	29.0	74.5	0.514	0.510
SWE 3.	110	641 (4520 kPa)	2.3	3937	34.9	99.7	0.573	0.569
SWE 4.	120	688 (4844 kPa)	2.4	3821	38.5	96.8	0.504	0.499
SWE 5.	130	735 (5168 kPa)	2.5	3673	42.0	93.0	0.444	0.439

[a] q Factor was determined from experiments for actual system.

soybean flakes. This optimum condition produced 3937 TIF µg/g $_{DSF}$ where the dielectric constant of the solution was 39.0 according to Yang et al. (1998). However, when water with further decreased dielectric constant at 120 °C and 688 psig (4844 kPa) was used, the amount of TIF extracted declined.

Table 4 also implies that all extraction conditions except temperature are insignificant. This result may influence the simulation-optimization technique applied to determine the optimum conditions. Consequently, extraction of SWE at low pressure is important. Fig. 4 shows a few low pressure SWE extractions at 110 °C over 2.3 h. The maximum amount of TIF was extracted at about 641 psig (4520 kPa). The amount of TIF decreased when the pressure was below 641 psig (4520 kPa).

Our results illustrate that extractions at temperatures other than 110 °C did not enhance the amount of TIF. The reason might be partially because water is less polar when T is below 110 °C and partially because TIF might be destroyed as T over 110 °C, indicated by a TGA analysis of the degradation temperature (around 140 °C) of one sample containing 37 wt.% TIF. At 110 °C, 641 psig (4520 kPa) was shown to be high enough to recover all five isoflavones from DSF.
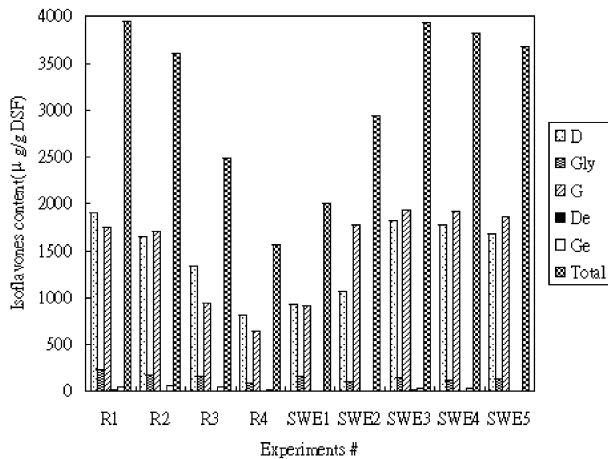
3.3. Purification

The four chromatograms of Fig. 2 imply that the isoflavone glycosides ($\sim$3800 µg) were much more abundant than their aglycones ($\sim$200 µg) in DSF. Most scientific studies reveal glycosides to have less biological effects than aglycones. Therefore, it is necessary to hydrolyze the extracts in order to transform glycosides to aglycones after the extracting process or to find a better way that can extract glycosides and do the transformation simultaneously.

Fig. 5 shows 49.0% TIF recovery in desorption (R_{des}), which produced a solid of 37.0% purity (P_{TIF}), in a case-A purification with 40 g SWE inputs (15153µg TIF). Figs. 6 and 7 separately present two scaling-up purifications, showing the de-sorption recovery and the purity of TIF of two mixed SWE extracts, obtained as case-B with 200g SWE inputs (10893 µg TIF) and case-C with 10,000 g SWE inputs (163,010 µg TIF). Purifying five isoflavones, using liquid–liquid extraction, produced only a solid that contained 24.3% TIF. The results of above experiments imply that producing a high purity of TIF might be significantly influenced by the feed concentration of TIF in the SWE extracts. Furthermore, using the SWE extract under optimum conditions of the feed solution is beneficial for purifying isoflavones.
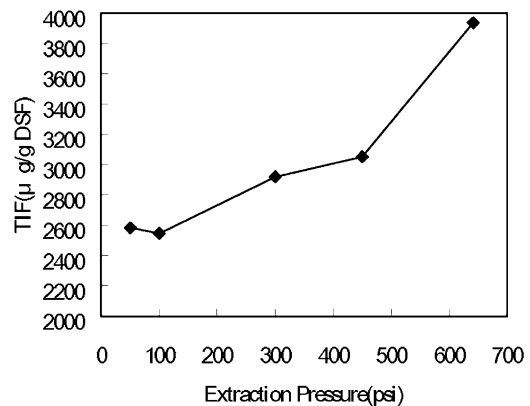

Fig. 2. The extracted amount of isoflavones by using various extractive condition (SWE3 was the optimum conditions for SWE).

Fig. 4. Effect of pressure at 110 °C over 2.3 h of extraction.

Fig. 3. Relationship between experimental conditions and static dielectric constant (Thermodynamic and Transport Properties of Water and Steam Version 1.0, 1999).
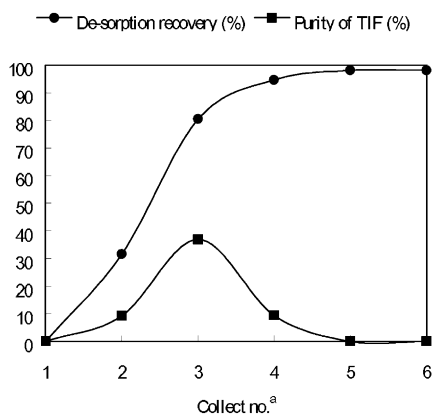
Fig. 5. Case A-solid phase purification (15 ml bed volume) with 40 g SWE input (15153 μg TIF). [a] Each fraction contains 10 ml of elute and fractions were collected continually.
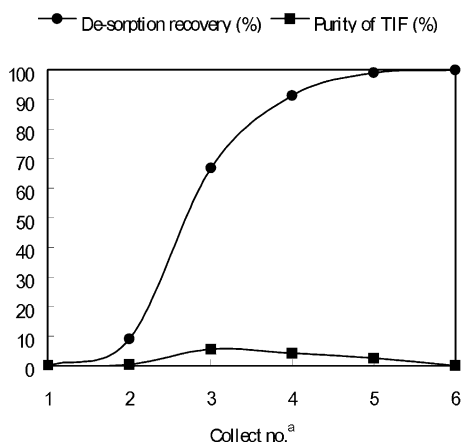

Fig. 6. Case-B solid phase purification (88 ml bed volume) with 200 g SWE input (10893 μg TIF). [a] Each fraction contains 50 ml elute and fractions were collected continually.
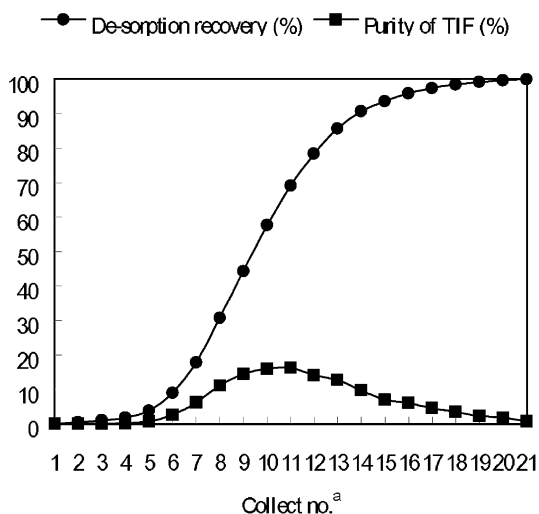

Fig. 7. Case-C solid phase extraction (230 ml bed volume) with > 10,000 g SWE input (163,010 μg TIF). [a] Each fraction contains 100 ml elute and fractions were collected continually.

4. Conclusion

This study demonstrates that superheated water extraction is a viable alternative means of extracting five important isoflavones from DSF and, also, an efficient way of recovering and purifying the resulting samples is by using solid-phase Amberlite XAD16-HP resin adsorption. The steepest ascent design for PWE experiments and the ascent step for SWE experiments revealed that the optimum conditions for SWE were 110 °C and 641 psig (4520 kPa) over 2.3 h of extraction. The decreasing dielectric constant at elevated temperature and pressure might play an important role for the enhanced extraction of TIF from DSF. Solid phase purification of the PWE (or SWE) sample of an aqueous solution easily produced a solid of 37% purity and 48.9% de-sorption recovery. Liquid–liquid extraction, using ethyl acetate, yielded a solid only with 24.3% TIF purity.

Acknowledgements

References

An-Erl King, V., Yueh-Ping Chang, M., & Kwo, S.-J. (1994). Studies on the gelation of soy protein induced by high pressure. *Journal of Agricultural and Food Chemistry*, *32*(3), 309.

Basile, A., J-Carmona, M. M., & Clifford, A. A. (1998). Extract of Rosemary by superheated water. *Journal of Agricultural and Food Chemistry*, *46*, 5205.

Coward, L., Barnes, N., Setchell, K. D. R., & Barnes, S. (1993). Genistein and daidzein, and their β-glucoside conjugates: Antitumor isoflavones in soybean foods from American and Asian diets. *Journal of Agricultural and Food Chemistry*, *41*, 1961.

Chandra, A., & Nair, M. G. (1996). Supercritical carbon dioxide extraction of daidzein and genistein from soybean products. *Phytochemical Analysis*, *7*, 259.

Chiang, W. D., Shih, C. J., & Chu, Y. H. (2001). Optimization of acid hydrolysis conditions for total isoflavones analysis in soybean hypocotyls by using RSM. *Food Chemistry*, *72*, 499.

Experimental designs (2000). ISBN 957-8324-12-X, Taipei, Taiwan.

Huang, C., & Hsieh, Y. (1998). Principle and application of soild-phase microextraction. *Chemistry (The Chinese Chem. Soc., Taipei)*, *56*(4), 311.

Murphy, P. A., Barua, K., & Hauck, C. C. (2002). Solvent extraction selection in the determination of isoflavones in soy foods. *Journal of Chromatography B*, *777*, 129.

Murphy, P. A., Song, T., Buseman, G., Barua, K., Beecher, G. R., Trainer, D., & Holden, J. (1999). Isoflavones in retail and institutional soy foods. *Journal of Agricultural and Food Chemistry*, *47*, 2697.

Nurmi, T., Mazur, W., Heinonen, S., Kokkonen, J., & Adlercreutz, H. (2002). Isoflavone content of the soy based supplements. *Journal of Pharmaceutical and Biomedical Analysis*, *28*, 1.

Nguyenle, T., Wang, E., & Cheung, A. P. (1995). An investigation on the extraction and concentration of isoflavones in soy-based products. *Journal of Pharmaceutical and Biomedical Analysis*, *14*, 221.

Rostagno, M. A., Araujo, J. M. A., & Sandi, D. (2002). Supercritical fluid extraction of isoflavones from soybean flour. *Food Chemistry*, *78*, 111.

Sanders, N. D. (1984). Visual observation of the solubility of heavy hydrocarbons in near-critical water. *Ind. Eng. Chem. Fundam*, *25*, 169.

Tani, T., Katsuki, T., Kubo, M., Arichi, S., & Kitagawa, I. (1985).

Chem. histocnemistry. VI. Tocopherols and isoflavones in soybeans (glycine max MERRILL, seeds). *Pharm. Bull.*, *33*(9), 3834.

Thermodynamic and Transport Properties of Water and Steam Version 1.0 (Base on the IAPWS-95 Formulation) (1999). ChemicaLogic Corporation, USA, www.chemicalogic.com.

Wang, H. J., & Murphy, P. A. (1994). Isoflavone content in commercial soybean foods. *Journal of Agricultural and Food Chemistry*, *42*(8), 1666.

Yang, Y., Belghazi, M., Lagadec, A., Miller, D. J., & Hawthorne, S. B. (1998). Elution of organic solutes from different polarity sorbents using subcritical water. *Journal of Chromatography A*, *810*, 149.