Original Article : Pharmaceutical Technology

Determination of Genistein and Daidzein in Soybeans under Various Extraction Methods by Developed High Performance Liquid Chromatography

การพัฒนาวิธีวิเคราะห์โดยใช้เครื่องโครมาโตกราฟีของเหลวสมรรถนะสูงของเจนิสเทอีนและไดเซอีนจากสารสกัดถั่วเหลืองด้วยวิธีการสกัดที่แตกต่างกัน

Parapat Sobharaksha, B. Pharm*; Ratana Indranupakorn, Ph.D**; Manee Luangtana-anan, Ph.D*

Sobharaksha P, Indranupakorn R, Luangtana-anan M. Determination of Genistein and Daidzein in Soybeans under Various Extraction Methods by Developed High Performance Liquid Chromatography. Thai Journal of Hospital Pharmacy 2011; 21(1):48-58.

The objective of this study was to develop a simple and reliable extraction technique that could be used routinely for extraction a broad range of concentrations of the isoflavones in soybeans and soy products. Five commonly extraction methods were chosen, i.e. soxhlet, shaker, stirring, ultrasonication, and vortexing. In order to simplify the analyses, a simple, specific and rapid high performance liquid chromatography (HPLC) method was used for the simultaneous determination of soy isoflavones from different extraction methods was developed and validated using genistein and daidzein, main essential isoflavones, as standards. Genistein and daidzein were baseline separated and quantitated on a C_{18} reversed phase column, using a gradient mobile phase composed of 0.1 percent acetic acid in deionized water and 0.1 percent acetic acid in methanol. The total run time was 30 minutes at a flow rate of 0.5-1.0 mL/min. Retention time was 13.8 and 14.9 minutes for genistein and daidzein respectively. The method was proven to be linear over genistein and daidzein concentrations range of 5 to 30 μg/mL with correlation coefficients of 0.9998 and 0.9999, respectively. Intra-and interassay CVs were 1.56 and 1.59 percent for genistein, and 1.61 and 1.59 percent for daidzein respectively. Mean recoveries were between 94.66 and 97.83 percent respectively. The developed method was successfully applied to quantitatively assay the soy isoflavones from the extraction of different methods. Among five different extraction methods, ultrasonication extraction exhibited a maximum yield of genistein and daidzein.

Key words: Isoflavones, extraction conditions, HPLC, soybeans

* Graduated Student, Pharmaceutical Technology Department, Faculty of Pharmaceutical Science, Silpakorn University, Nakornpathom Province

** Pharmacognosy Department, Faculty of Pharmaceutical Science, Huachiew Chalermprakiet University, Samutpakarn Province

ปารภัทร โศภารักษ์, รัตนา อินทรานุปกรณ์, มานี เหลืองธนะอนันต์. การพัฒนาวิธีวิเคราะห์โดยใช้เครื่องโครมาโตกราฟีของเหลวสมรรถนะสูงของเจนิสเทอีนและไดเซอีนจากสารสกัดถั่วเหลืองด้วยวิธีการสกัดที่แตกต่างกัน. วารสารเภสัชกรรมโรงพยาบาล 2554; 21(1):48-58.

วัตถุประสงค์ของงานวิจัยนี้เพื่อศึกษาถึงวิธีการสกัดและพัฒนาวิธีวิเคราะห์ปริมาณไอโซฟลาโวนจากถั่วเหลือง ในการทดลองนี้ ได้เลือกวิธีการสกัดห้าวิธีคือ การสกัดแบบซอกเลต การใช้เครื่องเขย่าแนวระนาบ การใช้เครื่องกวนแม่เหล็กไฟฟ้า การใช้คลื่นอัลตราโซนิค และการใช้เครื่องเขย่าผสมสาร เพื่อที่จะเปรียบเทียบหาวิธีที่สามารถสกัดได้สารสำคัญสูงสุด เพื่อนำมาเป็นตัวอย่างในการพัฒนาวิธีวิเคราะห์ด้วยเครื่องโครมาโตกราฟีของเหลวสมรรถนะสูง ให้ได้วิธีที่ง่าย สะดวก และรวดเร็วแก่การวิเคราะห์มากที่สุด โดยใช้สารที่เป็นองค์ประกอบหลักในไอโซฟลาโวน คือ ไดเซอีนและเจนิสเทอีนเป็นสารมาตรฐานในการวิเคราะห์ ใช้คอลัมภ์ชนิดรีเวิร์สเฟสซี 18 เฟสเคลื่อนที่ คือ 0.1 เปอร์เซ็นต์ของกรดอะซิติกในน้ำและ 0.1 เปอร์เซ็นต์ของกรดอะซิติกในเมทานอล ใช้เวลาในการวิเคราะห์ทั้งสิ้น 30 นาที ด้วยอัตราเร็ว 0.5-1.0 มิลลิลิตรต่อนาที รีเทนชันไทม์ (retention time) ของไดเซอีนและเจนิสเทอีน คือ 13.8 และ 14.9 นาที ตามลำดับ พร้อมทั้งทำการตรวจสอบความถูกต้องของวิธีวิเคราะห์ที่พัฒนาขึ้น ซึ่งได้ผลดังนี้ คือ ความแม่นยำของวิธีวิเคราะห์ได้ค่าความแปรปรวนของการทำวิเคราะห์ซ้ำภายในวันเดียวกันของไดเซอีนและเจนิสเทอีนเท่ากับ 1.61 และ 1.56 ตามลำดับ และค่าความแปรปรวนของการทำวิเคราะห์ซ้ำระหว่างวันของไดเซอีนและเจนิสเทอีนเท่ากับ 1.59 และ 1.59 ตามลำดับ สำหรับผลในการตรวจสอบความถูกต้องของวิธีวิเคราะห์ได้ทำการเพิ่มสารมาตรฐานลงในสารสกัดปริมาณ 75 เปอร์เซ็นต์, 100 เปอร์เซ็นต์ และ 125 เปอร์เซ็นต์ ได้ผล %recovery อยู่ในช่วง 94.66-97.83 ในการวิเคราะห์นี้ ได้ทำการเปรียบเทียบกับสารมาตรฐานเจนิสเทอีนและไดเซอีน โดยใช้ค่าความเข้มข้นระหว่าง 5-30 ไมโครกรัมต่อมิลลิลิตร สำหรับผลการทำ calibration curve ได้ค่า correlation coefficient ของไดเซอีนและเจนิสเทอีนเท่ากับ 0.9998 และ 0.9999 ตามลำดับ ดังนั้น ในการพัฒนาวิธีวิเคราะห์จากการศึกษานี้สามารถนำไปใช้เป็นวิธีที่เหมาะสมในการวิเคราะห์หาปริมาณไดเซอีนและเจนิสเทอีนจากสารสกัดถั่วเหลืองได้

คำสำคัญ: ไอโซฟลาโวน เจนิสเทอีน ไดเซอีน โครมาโตกราฟีชนิดเหลว ถั่วเหลือง

Introduction

In recent years, soybeans and soy based products have attracted increased attention owing to their nutritional and health-related beneficial aspects. Soy isoflavones are phytochemicals of prominent interest for some of these beneficial health effects.[1,2] In addition to isoflavones, soybeans contain a large number of bioactive phytochemicals such as phytosterols, protease inhibitors, and inositol hexaphosphates.[1-3] Isoflavones are a subclass of flavonoids and are also called phytoestrogen compounds due to their weak estrogen activity with potential protective effect against some hormone related diseases.[4,5] The main isoflavones found in soybeans are aglycones (genistein, daidzein, and glycitein) and their respective conjugated forms with glucose,

malonyl glucose, and acetyl glucose.[5-9] These three conjugated of genistein, daidzein, and glycitein are found in an approximately ratio of 6:3:1, respectively.[10] Several studies[10-13] have been shown that soy isoflavones play an important role in the reduction of cardiovascular disease risk as well as prevention of several hormonally influenced cancers, menopausal symptoms, and osteoporosis. Their abilities to act as antioxidants may also serve to prevent oxidative damage in living tissue. Earlier studies indicated that soy isoflavones, especially genistein, have the antiphotocarcinogenic properties by blocking both the initiation and promotion of skin carcinogenesis via the prevention of DNA adduct formation and inhibition of various oxidative events. Genistein can also significantly decrease UV-induced cutaneous erythrema and skin ulceration in human skin.[14-17] Further, isoflavones in soymilk can reduce hair growth and hair follicle dimensions.[18] These findings have encouraged soy isoflavones as possible topical alternative agent and surge of interest from the cosmetic industry.

Different researchers[3,4,10,11] have deployed various techniques such as stirring, shaker, pressurized liquid extractor, and supercritical fluid extractor for extraction of isoflavones from soybeans. All analyses were determined on different samples using a wide variation of solvent composition for extraction. There were reports of different yields obtained from different methods. However, none of the studies had investigated on the effect of various extraction techniques on the yield of isoflavones which were performed on one homogenous sample obtained by grinding soybeans procured from a single source. Thus, the comparison of the extraction methods has not been able to conclude. In addition, other studies[1,2,6,10,14,18] showed some drawbacks of quantification of diadzein and genistein due to long retention time and not simple solvents used. The objective of present investigation was, therefore to develop a simple extraction technique that was applicable for extraction a broad range of concentrations of the isoflavones in soybeans and soy products and to develop simple analysis method for the determination of only daidzein and genistein, the main isoflavones, used in neutraceutical preparation for human intake. The selected solvents for extraction were ethanol and isopropyl alcohol.

Materials and Methods

Plant Materials and Chemicals. The seeds of soybean [*Glycine max* (L.) Merr.] were acquired from a local producer in Nonthaburi province, Thailand (Rhai-Thip Co., Ltd). Two isoflavone standards, genistein and daidzein, were purchased from Sigma-Aldrich (St.Louis, MO). Isopropanol and ethanol (Merck-Darmstadt, Germany), which were the solvents for soybeans extraction, were of analytical grade. Methanol (Merck-Darmstadt,

Germany), solvent was used for HPLC analysis.

Sample Preparation. The dried soybean seeds were ground in a bench coffee grinder. The ground material was then passed through a standard mesh sieve (particle size<0.4287 mm), mixed thoroughly and stored in a freezer until extraction. Prior to extraction, the ground soybean seeds were defatted by isopropanol (10 mL/g of sample) using a magnetic stirrer (500 rpm) for 2 hours. After extraction, the mixture was centrifuged at 4500xg for 10 minutes and the supernatant was discarded. The remaining material was dried at room temperature under fume hood and used as sample for the experimental extraction.

Solvent Extraction Methods. The soybean isoflavones were extracted with 85% ethanol by using five different methods: soxhlet (A), shaker (B), stirring (C), ultrasonication (D), and vortexing extractions (E). All extraction methods were carried out with one gram of ground soybean seeds using the single extraction solvent (85% ethanol) and extraction time of 1 hour for all methods.

1. Extraction by Using a Soxhlet Apparatus. An exact amount of 10 grams of ground soybean seeds was placed in a thimble inside soxhlet extraction apparatus. The extraction was carried out using 85% ethanol as solvent (10 mL/g of sample) for 1 hour. The crude extract was concentrated by reduced pressure evaporation (45°C) and then centrifuged at 2140xg for 10 minutes. The supernatant was taken into 10 mL volumetric flask. Two millilitres of the extract was filtered through 0.45 μm PTFE (polytetrafluoroethylene) syringe filter for isoflavone analysis by using HPLC (sample A). Three replicate HPLC analyses of the extract were carried out.

2. Extraction by Using a Shaker, Stirring, Ultrasonication, and Vortexing Procedures. In each method, 1-g of sample was weighed and 10 mL of 85% ethanol was added to each flask. For shaker, sample was vigorously shaken on a horizontal shaker (HS 501 digital, Kika Labortechnik, Staufen, Germany) at a high speed for 1 hour (sample B). For stirring, extraction was carried out by placing extraction flask on a magnetic stirrer (Heidolph hot plate magnetic stirrers, Kelheim, Germany) for 1 hour (sample C). Extraction with ultrasonication, sample flask was placed in a ultrasonic bath (Branson 3510, Ultrasonic Corporation, Danbury, CT, USA) for 1 hour (sample D). For vortexing procedure, 20 mL tube with sample was placed on a Daigger Vortex Genie 2 (Scientific Industries, Inc., NY, USA) for 1 hour (sample E).

The crude extract from each procedure was centrifuged at 2140xg for 10 minutes. The supernatant was taken into 10 mL volumetric flask. Two millilitres of the extract was filtered through 0.45 μm PTFE syringe filter for isoflavone analysis by using HPLC. Three

replicate HPLC analysis of each extract were carried out for each sample.

Identification and Quantification of Soy isoflavones by Using Developed High Performance Liquid Chromatographic (HPLC) Method. Analyses were carried out by HPLC. A Finnigan modular LC system with a Model P4000 dual pump equipped with a Rheodyne 7725i injector linked to a 20 μL loop and a Model UV 6000 photodiode array detector was used for analysis by liquid chromatography. A Phenominex C18 column (250x4.6 mm I.D., particle size 10 μm) was used for chromatographic separations. The chromatographic data were obtained by a professional component (PC) system, and a software ChromQuest from Thermo Fisher Scientific was used to acquire and process the data. The extracted solutions obtained were analysed by HPLC. Gradient elution was needed for complete separation of the analysis. The mobile phase consisted of two eluents: (A) 0.1 percent acetic acid in deionized water and (B) 0.1 percent acetic acid in methanol. The optimized gradient elution program is shown in Figure 1.

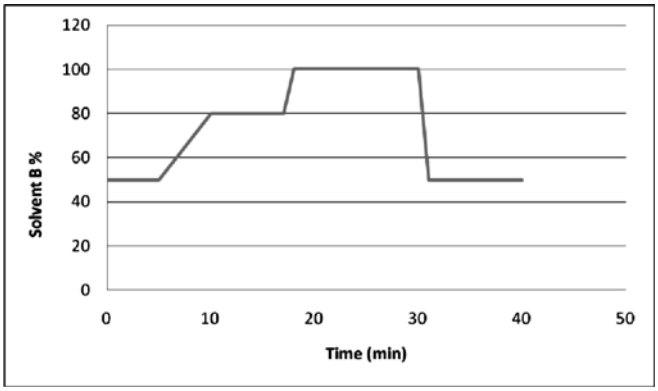

Figure 1. Gradient profile in the HPLC analysis of soy isoflavones

The system was maintained at 50 percent (B) for 5 minutes with the flow rate of 1 mL/min, then, increased to 80 percent in 5 minutes with the flow rate of 0.5 mL/min and held at 80 percent for another 7 minutes with the flow rate of 1 mL/min. At the end, the system was set to increase solvent (B) from 80 to 100 percent within 2 minutes, holding these conditions for 11 minutes and then returned to the original condition, 50 percent (B), for 10 minutes. Total run time 40 minutes including 10 minutes stabilization time. The chromatographic analysis was performed at an ambient temperature and detection wave-length at 254 nm. Injection volume was 20 μL.

The stock solutions of genistein and daidzein were dissolved in methanol and dimethylsulfoxides respectively. These solutions

were diluted to a final concentration between 5 and 30 μg/mL with the same solvents and analyzed by HPLC. The identification of the separated compounds in soybean extracts was assigned by a comparison of retention times and co-chromatogram with authentic standards. Quantification was carried out by integration of the peak areas using the external standard method. Calibration curves were made for each standard with five different concentrations at 5.00, 8.00, 10.00, 20.00, and 30.00 μg/mL and observed good linear relationships for all the calibration curves. The extracted samples obtained from various extraction procedures were quantitatively analyzed one by one. The best extraction procedure was then selected the ultrasonicate method for validation test.

Statistical Analysis. All statistical analyses were conducted using ANOVA (α=0.05) and Scheffe using a Statistical Package for the Social Sciences software (SPSS version 16 for windows from SPSS Inc., Chicago, Illinois, USA).

Results and Discussions

1. Identification of Isoflavones in Soybeans from Various Extraction Methods by HPLC. The HPLC chromatogram of the isoflavone standards, genistein, and daidzein, is represented in Figure 2 (a). The use of a Pheno minex C18 column with gradient elution consisted of 0.1% acetic acid (A) and 0.1% acetic acid/methanol (B) as binary mobile phase, resulted in a good resolution of standards about within 30 minutes. The chromatograms of the isoflavones extracted from ground soybean seeds by using different methods, i.e. soxhlet, shaker, stirring, ultrasonication, and vortex (sample A-E) showed differences in the isoflavone profile [Figure 2(b)-2(f)]. Genistein and daidzein obtained from samples were identified by comparison of retention times with pure standards, as well as by photodiode array detection and spiking the standard component in the extract. The retention times were 13.8 and 14.9 minutes for genistein and daidzein respectively. Results indicated that the developed HPLC could be used to identify genistein and daidzein.

2. Quantification of Isoflavones in Soybeans from Various Extraction Methods by HPLC. The extraction efficiency of soybean isoflavones with single extraction solvent mixture (85 percent ethanol in deionized water) was carried out by using five commonly extraction methods (soxhlet, shaker, stirring, ultrasonication, and vortex). The contents of individual isoflavones (genistein and daidzein) were calculated from peak areas of compounds are listed in Table 1, A-E. The chromatograms of the isoflavones standard and the extracts from ground soybean seeds by using different methods showed differences in the isoflavone profile [Figure 2(a)]. Results indicated that optimum yields of genistein and daidzein were

obtained with ultrasonication procedure, which showed the amounts of 272.27±6.83 and 343.53±6.18 μg/g respectively; while the lowest yields of genistein (101.45±5.75 μg/g) and daidzein (165.64±.89 μg/g) were obtained by using soxhlet and vortexing procedures respectively. The highest yield of ultrasonication was the result of higher frequency of ultrasound causing the penetration onto water (liquid mediums). This had a potential to cause stream waves and bubbles called "*cavitation*". Cavitation is the phenomenon of formation of very low size air bubbles, approximately 1 in 1 million meter (micron), produced by a flowing liquid, generating high power of energy, accordingly.[19] The production of highest energy allowing greater penetration of solvent into the sample matrix, in comparison to other techniques, was possibly attributed to the optimum isolation of essential substances from soybean. The developed HPLC method, hence, could be proved to determine the essential substances of soybean obtained from various extraction methods.

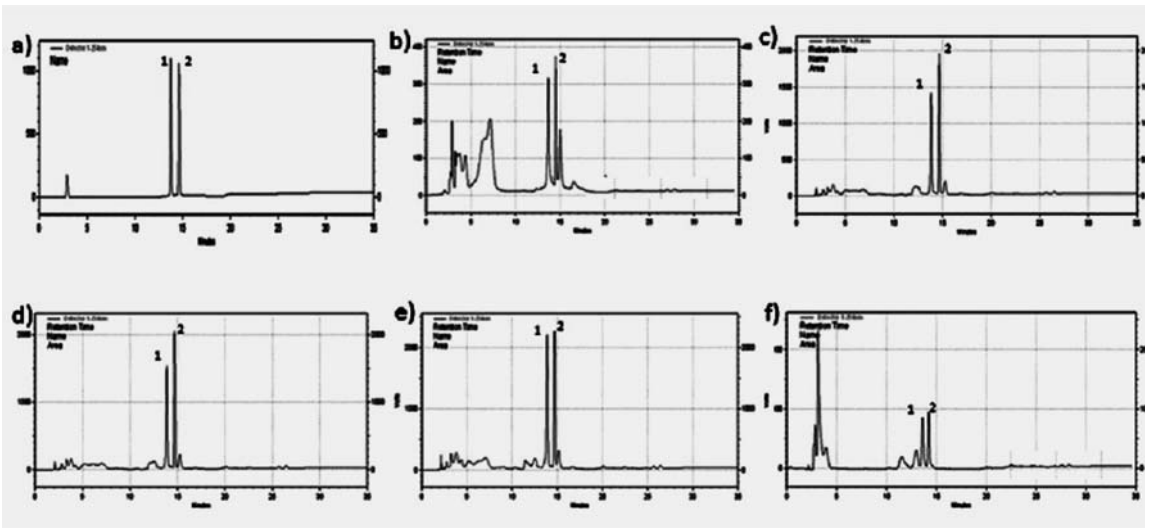

Figure 2. RP-HPLC chromatograms of isoflavones, standard mixture (a) genistein (1) and daidzein (2); isoflavones extracted from ground soybean seeds by using soxhlet (b), shaker (c), stirring (d), ultrasonication (e), and vortexing procedures (f).

Values were expressed as means±SD (n=3); values differing at $p \leq 0.05$ were mostly considered significant with each other by least significant difference test.

3. Validation of Analytical Method. Isoflavones obtained by ultrasonication method was used for validation study. The accuracy of the method was evaluated by recovery assay,

Table 1. Amount of isoflavones, genistein and daidzein, extracted soybean seeds using different extraction methods

Extraction Methods	Genistein (μg/g)	CV	Daidzein (μg/g)	CV
Soxhlet (A)	101.45±5.75	5.67	186.11±6.31	3.39
Shaker (B*)	242.24±8.42	3.48	235.48±10.22	4.34
Stirring (C*)	248.69±5.12	2.06	251.01±3.54	1.41
Ultrasonication (D)	272.27±6.83	2.51	343.53±6.18	1.80
Vortex (E)	173.27±5.65	3.26	165.64±0.89	0.54

*Only shaker and stirring methods were not significantly different

CV = coefficient of variation, μg/g = microgram/gram

adding known amounts of each isoflavone standard to a known amount of sample D (extraction by ultrasonication), to obtain three different levels (75, 100, and 125 percent) of addition. Spiked amounts ranged from 100.41-167.35 μg/g for genistein and 106.27-177.12 μg/g for daidzein. Each sample was analysed quantitatively in triplicate. The mean recovery and %RSD (relative standard deviation) were calculated. The expected values for isoflavones and the recoveries for each level are summarized in Table 2. Average recoveries of spiked isoflavones were between 94.72 and 97.84 percent for genistein and daidzein, respectively. No considerable differences had been found between recoveries at different spiked levels, which indicated good accuracy of the method. The obtained results of genistein and daidzein had shown recoveries between 90-107 percent, within the range of the Association of Official Agricultural Chemist (AOAC) guidelines.[20]

To assess the precision of the method, six replicates of the sample D were determined on the same day (intraday precision) and one time injection on five consecutive days (inter-day precision). The results showed acceptable precision with the developed HPLC method as revealed by coefficient of variation (CV) data presented in table 2. Intra- and interassay CVs were 1.56 and 1.59 percent for genistein and 1.61 and 1.59 percent for daidzein, respectively, with no differences in CVs between compounds at high or low concentrations. The data showed ±2.0 %RSD, therefore, complying with the evaluation criterion of the AOAC guidelines.[20]

The linearity was determined using five concentrations of both standard solutions of genistein and daidzein in the range of 5-30 μg/mL (n=3). The regression equations were found by plotting the peak area (y) versus the isoflavone concentration (x) expressed in μg/mL. The result showed good linearity with

calibration curves for genistein and daidzein were $Y = 6.0806 \times 10^8 + 354440$ and $Y = 4.8209 \times 10^8 + 200815$, respectively. The correlation coefficient (r^2) for genistein and daidzein was 0.9998 and 0.9999, respectively, as shown in Table 3 and Figures 3. The (r^2) demonstrated the excellent relationship between peak area and concentration of each isoflavone standard.

Table 2. Accuracy and precision of the developed HPLC method

		Accuracy				Precision	
Isoflavone	Concentration (μg /g)	Added (μg /g)	Found (μg/g)	Recovery (%)	CV	Intraassay CV (n=6)	Intraassay CV (n=6)
Genistein	133.88	100.41	221.75	94.65	3.23	1.56	1.59
		133.88	265.78	99.26	1.09		
		167.35	271.30	90.06	2.27		
Daidzein	141.69	106.27	247.51	99.82	0.21	1.61	1.59
		141.69	284.11	100.25	6.15		
		177.12	297.90	93.44	0.49		

μg /g = microgram/gram, CV = coefficient of variation; n = number of repeated analysis

Table 3. Concentrations and peak areas of standard isoflavones; genistein and daidzein, to illustrate linearity

	Concentration	Peak Area					
Isoflavones	(μg /g)	n1	n2	n3	Average	CV	R^2
Genistein	5.00	3396729	3361784	3340710	3366408	0.8405	0.9998
	8.00	5115110	5127858	5133449	5125472	0.1834	
	10.00	6345273	6600104	6619991	6521789	2.3489	
	20.00	12393783	12975419	12485599	12618267	2.4780	
	30.00	18466908	18595508	18522613	18528343	0.3480	
Daidzein	5.00	2611420	2701351	2642957	2651909	1.7206	0.9999
	8.00	4031109	4059774	4120455	4070446	1.1207	
	10.00	4996506	4862336	5126701	4995181	2.6463	
	20.00	9900767	10031130	9363960	9765286	3.6211	
	30.00	14677211	14752713	14710353	14713426	0.2572	

μg /g = microgram/gram, CV = coefficient of variation, R = correlation coefficient, n = number of analysis

Conclusions

The isoflavone extraction methods presented in this article has been developed by using one simple single step and avoiding the use of special technique that could be used routinely for extraction a broad range of concentrations of the isoflavones in soybeans and soy products.The most suitable extraction method for isoflavones proved to be the ultrasonication. The developed HPLC method for

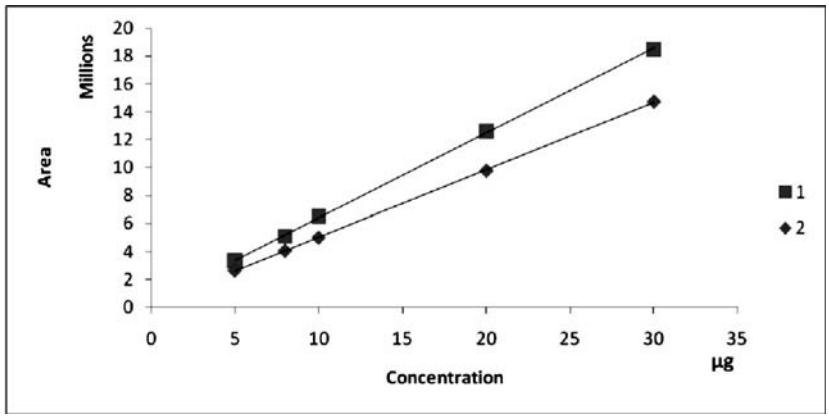

Figure 3. The calibration curves of standard genistein (1) and daidzein (2) by a HPLC system

quantification of genistein and daidzein has been validated and shown to be reliable, accurate, precise, and linear (in the concentration range of 5-30 μg/mL). The validity of the method has met the requirement of AOAC guidelines. Therefore it can be used as an accurate routine procedure for the quantification of genistein and daidzein in soybeans and soy products with short retention times (13.8 and 14.5 minutes respectively) and simple.

References

1. Rostagna MA, Araujo JMA, Sandi D. Supercritical fluid extraction of isoflavones from soybean flour. Food Chem 2002; 78: 111-7.
2. Rostagno MA, Palma M, Barroso CG. Solid-phase extraction of soy isoflavones. J Chromatogr A 2005; 1076: 110-7.
3. Georgetti SR, Casagrande R, Souza CRF, et al. Spray dry of the soybean extract: effects on chemical properties and antioxidant activity. LWT 2008; 41: 1521-7.
4. Rostagno MA, Palma M, Barroso CG. Microwave assisted extraction of soy isoflavones. Anal Chim Acta 2007; 588: 274-82.
5. Pe?alvo JL, Nurmi T, Adlercreutz H. A simplified HPLC method for total isoflavones in soy products. Food Chem 2004; 87: 297-305.
6. Rostagno MA, Palma M, Barroso CG: Ultrasound-assisted extraction of soy isoflavones. J Chromatogr A 2003; 1012: 119-28.
7. Kao T, Chien J, Chen B. Extraction yield of isoflavones from soybean cake as affected. Food Chem 2007; 107: 1728-36.
8. Yu J, Liu Y, Qiu A, et al. Preparation of isoflavonesenriched soy protein isolate from defatted soy hypocotyls by supercritical CO_2. LWT 2007; 40: 800-6.
9. Rostagno MA, Palma M, Barroso CG. Short-term stability of soy isoflavones extracts: sample conservation aspects. Food Chem 2005; 93: 557-64.
10. Rostagno MA, Palma M, Barroso CG. Pressurized liquid extraction of isoflavones from soybeans. Anal Chim Acta 2004; 522: 169-77.
11. Liggins J, Bluck LJC, Coward WA, et al. Extraction and quantification of daidzein and genistein in food. Anal Biochem 1998; 264: 1-7.
12. Achouri A, Boye JI, Belanger D. Soybean isoflavones: efficacy of extraction conditions and effect of food type on extractability. Food

Research Inter 2005; 38: 1199-204.

13. Dixon RA, Ferreira D. Genistein. Phytochemistry 2002; 60: 205-11.
14. Kim S, Kim S, Lee J, et al. Protective effects of dietary soy isoflavones against UV-induced skin-aging in hairless mouse model. J Am Coll Nutr 2004; 23(2): 157-62.
15. Draelos, ZD. Skin lightening preparations and the hydroquinone controversy. Dermatol Ther 2007; 20: 308-13.
16. Chiu A, Kimball AB. Topical vitamins, minerals and botanical ingredients as botanical ingredients as modulators of environmental and chronological skin damage. Br J Dermatol 2003; 149: 681-91.
17. Lazarus MC, Baumann lS. The use of cosmeceutical moisturizers. Dermatol Ther 2001; 14: 200-7.
18. Seiberg M, Liu JC, Babiarz L, et al. Soymilk reduces hair growth and hair follicle. Exp Dermatol 2001; 10: 405-13.
19. Siriwan S, Nanthana C, Patcharaporn P, et al. Improved extraction method for evaluate and andrographolide in fahtalaijone using sonication. Agricultural Sci J 2006; 37(6): 419.
20. Ruengvisaet N. Method validation. In: Sripa K, Hemintaraporn J, Ratanavijit P, et al, ed. Good Laboratory Practice: Drug Quality Assurance. Bangkok: Mahidol University Press, 2005: 95-101.