

Analytical, Nutritional and Clinical Methods

Comparison of extraction solvents and techniques used for the assay of isoflavones from soybean

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Abstract

The impact of extraction solvents and techniques on the assay of isoflavones from soybean was investigated. This systematic study was undertaken to address substantial variations in the solvents and procedures used for the extraction of isoflavones from soybeans by different research groups as described in the recent peer-reviewed published literature. Comparison of four previously optimized and commonly deployed solvent mixtures (acetonitrile:water 58:42 (v/v); ethanol:water 70:30 (v/v); methanol:water 90:10 (v/v); superheated pressurized water) was carried out for the extraction of isoflavones. In addition, we also examined the extraction efficiencies of three additional new solvent mixtures (dimethyl sulphoxide:acetonitrile:water, 5:58:37 (v/v/v); dimethyl sulphoxide:ethanol:water, 5:70:25 (v/v/v); Genapol:water 5:95 (v/v)) for the extraction of isoflavones from soybeans. Assessments of six commonly used extraction techniques (shaking, vortexing, sonication, stirring, Soxhlet, and pressurized liquid extraction (PLE)) with an optimized extraction solvent mixture was also performed. Both, the total isoflavones content and the isoflavones HPLC profile varied significantly with different extraction solvents and techniques. Optimum total isoflavones recoveries from soybean samples were obtained with dimethyl sulphoxide:ethanol:water (5:75:25, v/v/v) solvent mixture using a PLE. Intermediate extraction recoveries of total isoflavones from soybean samples were obtained with the other extraction solvent mixtures and techniques tested. The extraction efficiencies of isoflavones with shaker, vortex, stirring, and Soxhlet were between 65% and 70% as compared to PLE. Total isoflavones extracted by the sonication procedure was 93.3% as compared to PLE.

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Keywords: Soybeans; Isoflavones; Extraction solvent optimization; Stirring; Shaking; Vortexing; Pressurized liquid extraction; Sonication; HPLC

1. Introduction

Interest in soybeans and soy based products has grown significantly in the past few decades due to the association of soybean consumption with variety of health protective effects (Hasler, 1998). Soybeans are known to contain a large number of bioactive phytochemicals such as isoflavones, saponins, phytosterols, protease inhibitors, inositol hexaphosphates, sphingolipids, phenolic acids, and Bowman-Birk trypsin inhibitors (Kennedy, 1998; Koratkar &

Rao, 1997; Lee, Wang, Murphy, & Hendrich, 1995; Slavin, Jacobs, & Marquart, 1997; Vesper et al., 1999). Isoflavones enriched extracts have been evaluated in the prevention of a wide range of health problems associated with menopause, cardiovascular disease, osteoporosis, and in breast, prostate, and colon cancers (Anderson & Garner, 1997; Caragay, 1992; Messina, 1999). Isoflavones are widely distributed in the plant kingdom, but accumulate predominantly in plants of the *Leguminosae* family. The best natural source of isoflavones is soybeans, which have been a major part of the traditional diet for eastern Asian populations for centuries. The global annual consumption of soybeans has increased from 114 to 170 million tons during the past decade (Klejdus et al., 2004). Soybeans contain

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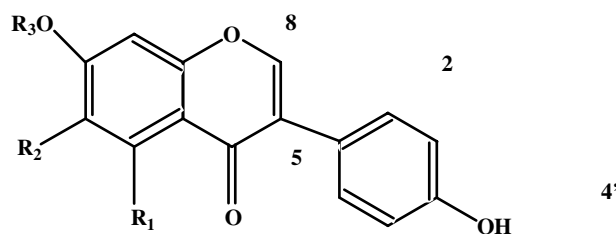
1.2–2.4 mg of total isoflavones per gram of sample (Rostagno, Palma, & Barroso, 2004). This hundred percent variation in isoflavones content in soybeans is due to variation in genotypes, environment, location, post harvest storage, and assay procedures.

Isoflavones are oxygen heterocycles containing a 3-phenylchroman skeleton that is hydroxylated at 4' and 7 positions (Penalvo, Nurmi, & Adlercreutz, 2004) (Fig. 1). Based on the substitution pattern on carbons 5 and 6, three aglycon forms of isoflavones commonly found in soybeans are daidzein, genistein, and glycitein. These three isoflavones can also exist in conjugated forms with glucose (daidzin, geinstein, glycitin), malonylglucose (malonyldaidzin, malonylgeinstein, malonylglycitin), and acetylglucose (acetyldaidzin, acetylgeinstein, acetylglycitin) units. Thus 12 free and conjugated forms of isoflavones have been isolated from different soybean samples (Fig. 1).

As 12 different soy isoflavones have a wide variation in polarities, development of an optimized extraction procedure for all isoflavones has been a challenging task. Different researchers have deployed various solvents and techniques for extraction of isoflavones from soybeans. The data presented in Table 1 summarizes some of the most popular methods for extraction of isoflavones used by various research groups found in peer-reviewed literature during the past five years. Researchers have utilized different extraction equipment or techniques such as multi-wrist shaker, rotary shaker, stirring, sonicator, Soxhlet, supercritical fluid extractor, and pressurized liquid extractor for extraction of isoflavones from soybeans (Table 1). In addition, a wide variation in extrac-

tion solvent composition (methanol, ethanol, and acetonitrile with different proportions of acidified and non-acidified water) has been used. Rostagno et al. (2004) compared the extraction efficiency of isoflavones with different ethanol and methanol water mixtures (30–80%) using a pressurized liquid extractor (PLE). They reported that optimum yields of isoflavone content were obtained with 70% ethanol in water. In another study, Klejdus et al. (2004) reported that methanol:water (90:10%, v/v) was the best solvent mixture for extracting isoflavones from soybean samples. At about the time, Lin and Giusti (2005) compared the extraction efficiencies of different aqueous (acidified and non-acidified) mixtures of methanol and acetonitrile solvent mixtures and reported that 58% non-acidified acetonitrile provided optimum extraction of isoflavones. Thus wide variations in the usage of different solvent mixtures and techniques have been applied for the extraction of isoflavones from soybeans and other plant matrices.

The amounts of isoflavones in plants, fruits, vegetables and herbs are influenced by genotypes, agronomic practices, maturity at harvest, and post-harvest, storage, climatic, regional, and processing conditions (Achouri, Boye, & Belanger, 2005; Griffith & Collison, 2001; Kao, Su, & Lee, 2004; Klejdus et al., 2005; Lee et al., 2004; Murphy, Barua, & Hauck, 2002; Wu, Wang, Sciarappa, & Simon, 2004). To minimize these variations and to evaluate the influence of sample preparation (extraction solvents and techniques), all analyses were performed on one homogenous sample obtained by grinding soybeans procured from a single source.



Name	R ₁	R ₂	R ₃
Daidzein	H	H	H
Glycitein	H	OCH ₃	H
Genistein	OH	H	H
Daidzin	H	H	Glu
Glycitin	H	OCH ₃	Glu
Genistin	OH	H	Glu
Acetyldaidzin	H	H	Glu-COCH ₃
Acetylglycitin	H	OCH ₃	Glu-COCH ₃
Acetylgenistin	OH	H	Glu-COCH ₃
Malonyldaidzin	H	H	Glu-COCH ₂ COOH
Malonylglycitin	H	OCH ₃	Glu-COCH ₂ COOH
Malonylgenistin	OH	H	Glu-COCH ₂ COOH

Fig. 1. Chemical structures of 12 isoflavones isolated from soybeans.

Table 1
Summary of commonly deployed procedures used by various authors for extraction of isoflavones soybean samples

Serial #	Extraction procedure	Best solvent	Publication year	Reference
1	Ground manokin soybeans (2 g) were extracted with 12 mL of six different extraction solvents (83% ACN, 83% acidified ACN, 80% MeOH, 80% acidified MeOH, 58% ACN, and 58% acidified ACN). Extractions of isoflavones were carried out at RT for 2 h	58% ACN	2005	Lin & Giusti
2	Soy meal was extracted with acidified ACN (58%) by shaking sample for 2 h at RT	One solvent	2004	Lee et al.
3	Comparison of four different sample preparation (extraction and hydrolysis) procedures with three different solvents (80% EtOH, 80% acidified EtOH, and 80% MeOH) was carried out at various temperature (60–100 °C) for different time intervals (30–240 min)	80% EtOH containing 1 M HCl	2004	Penalvo et al.,
4	Two grams of different food samples were extracted with four different acidified solvents 53% ACN, 53% MeOH, 53% EtOH and 53% acetone. The mixture was stirred at room temperature for 2 h	53% ACN	2002	Murphy et al.,
5	Defatted soy samples were extracted with 80% ACN, 80% MeOH and 80% EtOH by stirring and sonicating samples. The authors suggest replicate extraction are essential for optimum extraction. Significant decrease in isoflavone yields were obtained with one extraction cycle	Similar yields with all three solvents	2005	Achouei et al.,
6	Freeze dried soybeans were extracted with pressurized liquid extractor (PLE) with different proportions of (30–80%) of aqueous EtOH and MeOH solvent mixtures at different temperature, pressures, solid-to-solvent ratios. Optimization of PLE conditions are described	70% EtOH	2004	Rostango et al.,
7	The authors used a high pressure hot water process to extract five isoflavones from deflated soy flakes An experimental design was adopted to optimize extraction conditions, and five isoflavones were purified. The optimum conditions were determined as 110 °C and 641 psig over 2.3 h of extraction	One solvent	2004	Li-Hsun et al.,
8	Comparison of different extraction conditions (extraction time, temperature, pressure, cycles, solvents) and techniques (PLE, PLE + sonication, sonication, and Soxhlet)	90% MeOH	2004 and 2005	Klejdus et al.,

This paper provides a direct comparison of four commonly used extraction solvents or solvent mixtures (acetonitrile:water (58:42, v/v); ethanol:water (70:30, v/v); methanol:water (90:10, v/v); superheated pressurized water) used for the extraction of isoflavones from soybeans as documented in the peer reviewed literature (Table 1). We have also examined efficiencies of three additional solvent mixtures (dimethyl sulphoxide:ethanol:water (5:70:25, v/v/v), dimethyl sulphoxide:acetonitrile:water, (5:58:37, v/v/v) and 5% Genapol, a nonionic surfactant oligo (ethylene-glycol) monoalkyl ether in water) for extraction of isoflavones from soybeans. In addition, we have compared extraction efficiencies of six commonly used extraction techniques (stirring, Soxhlet, sonication, PLE, vortexing and mechanical wrist shaker) for the extraction of isoflavones from soybeans.

2. Materials and methods

2.1. Plant material

Soybean seeds were purchased from a local grocery store (Mom in Beltsville, Maryland). Immediately, after receipt, the material was ground in a home style coffee grinder. The ground material was then passed through a standard 20-mesh sieve (particle size <0.825 mm). The ground sample was mixed thoroughly and subdivided into multiple

aliquots in amber bottles. Each bottle was flushed with nitrogen, and stored in a freezer (<−60 °C) until analyzed.

2.2. Chemicals

HPLC grade acetonitrile and methanol were purchased from Fisher Chemicals (Fair Lawn, NJ, USA), and denatured anhydrous ethanol was obtained from Mallinckrodt (Paris, KY, USA). HPLC-grade formic acid and dimethyl sulphoxide was procured from Aldrich Chemical Company (Milwaukee, WI, USA). Diatomaceous earth (Celite 545) and Ottawa sand were purchased from Fisher Chemicals (Fair Lawn, NJ, USA). Deionized water (18 Ω) was prepared using a Millipore Milli-Q purification system (Millipore Corp., New Bedford, MA, USA). Genapol X-080 was purchased from Sigma–Aldrich Chemie GmbH (Munich, Germany). Polyvinylidene difluoride (PVDF) syringe filters with pore size 0.45 μm were purchased from National Scientific Company (Duluth, GA, USA). Isoflavone standards (Genistein, Daidzin, Genistin, Daidzein, and Glycitin) were purchased from LC Laboratories (Woburn, MA, USA).

2.3. Comparison of extraction solvents

All extractions for the comparison of extraction efficiencies of different solvent mixtures were carried out with an

Accelerated Solvent Extractor (ASE) from Dionex Corporation (Model ASE 200, Dionex Corporation, Sunnyvale, CA, USA). Aliquots of 500 ± 1 mg of ground soybean seeds were placed in an 11 ml stainless steel extraction cell. Two circular cellulose filters (size 1.983 mm, Dionex Corporation) were placed at the bottom of the extraction cell in order to prevent suspended particles from entering the collection vials. The remaining void volume in the cell was filled with Ottawa Sand. Both extraction cells and extract collection amber vials were arranged appropriately in the two designated carousels. Extractions were carried out using seven different solvent mixtures 1. acetonitrile:water (58:42, v/v); 2. dimethyl sulphoxide:acetonitrile:water, (5:58:37, v/v/v); 3. ethanol:water (70:30, v/v); 4. methanol:water (90:10, v/v); 5. dimethyl sulphoxide:ethanol:water, (5:70:25, v/v/v); 6. water; and 7. genapol:water (5:95, v/v). Extractions were performed under previously optimized conditions as reported by Rostagno et al. (2004). In short, all extractions were carried out at 1000 psi at 100 °C, with a 5 min equilibration time, a 7 min static time, and a 90 s purge time for each extraction cycle. A total of three extraction cycles were performed for each sample. The extraction temperature was set to 115 °C only in the case when water was the extraction solvent. The extracts were collected in 60 ml amber sample vials fitted with Teflon coated rubber caps (I-CHEM, New Castle, DE, USA). Each extract was transferred to a 25 ml volumetric flask and the total volume was adjusted to 25 ml with the appropriate extraction solvent mixture. These aliquots of soybean extracts were filtered through a 0.45 µm PVDF syringe filter prior to analysis of isoflavones by HPLC assay. Four replicate extractions and duplicate HPLC analyses of each extract were carried out for each sample.

2.4. Comparison of extractions techniques

Comparison with five other extraction techniques (vortexing, shaking, stirring, sonication and Soxhlet) commonly used for extraction of isoflavones from soybean samples was carried out using the single optimized extraction solvent mixture (dimethyl sulphoxide:acetonitrile:water, (5:58:37, v/v/v)) with the same solid-to-solvent ratio.

2.4.1. Extraction of isoflavones from soybean samples by stirring, shaker, sonication, and vortexing procedures

Approximately 500 ± 1 mg of ground soybean sample were placed in 16×125 mm screw cap vial and 8 ml of extraction solvent mixture was added to each tube. Stirring was carried out by adding one 8×1.5 mm PVDF coated magnetic bar to each vial and placing vials in a 100 ml beaker on a Corning hot plate/stirrer (Model PC 351). For shaker, extractions were carried out by placing extraction vials containing ground soybean sample with 8 ml extraction solvent on a Burrell Wrist Shaker (Model 75, Burrell Corporation, Pittsburg, PA, USA) at a high speed (setting # 10) for 60 min. For sonication, sample vials were placed

in a sonicator bath (Branson 2510, Branson Ultrasonic Corporation, Danbury, CT, USA) at ambient temperature for 15 min. Extraction with vortexing was performed by vortexing the vials for 1 min (three times with each cycle) on a Daigger Vortex Genie 2 (Scientific Industries, Inc., Bohemia, NY, USA). After extraction with the different procedures, the mixture was centrifuged at a low speed (838g) for approximately 10 min. The supernatant was transferred into a 25 ml volumetric flask. The residue was re-suspended in the fresh extraction solvent mixture (8 ml dimethyl sulphoxide:acetonitrile:water (5:58:37, v/v/v)) each time and the extraction process was repeated twice. A total of three extraction cycles were performed with each procedure. The supernatant from the three extraction cycles were pooled and the volume of the combined extract was adjusted to 25 ml with extraction solvent and appropriate aliquots of extracts were filtered through a 0.45 µm PVDF syringe filter for isoflavone analysis by HPLC analysis. Four replicate extractions and duplicate HPLC analyses of each extract were carried out for each sample.

2.4.2. Soxhlet extraction of isoflavones from soybean samples

Soxhlet extractions were carried out by placing ground soybean sample ($3 \text{ g} \pm 1 \text{ mg}$) in a Whatman filter paper # 4. The filter paper containing sample was wrapped and placed in a thimble. The thimble was then placed in a Soxhlet extraction apparatus. Approximately 125 ml of extraction solvent (8 ml dimethyl sulphoxide:acetonitrile:water (5:58:37, v/v/v)) was added into the Soxhlet apparatus. All samples were extracted with hot boiling solvent mixture for 3 h. After 3 h, extraction apparatus was rinsed twice with additional 12.5 ml extraction solvent mixture. The total volume of the soybean extract was adjusted to 150 ml. Appropriate aliquots of extracts were filtered through a 0.45 µm PVDF syringe filter for isoflavone analysis by HPLC analysis. Four replicate extractions and duplicate HPLC analyses of each extract were carried out for each sample.

2.5. Determination of phenolic compounds by HPLC

Analysis of isoflavones from all extraction procedures and seven different extraction solvents was carried out using an Agilent 1100 HPLC system consisting of a binary pump with a vacuum degasser, a thermostatted column compartment, an auto-sampler, and a diode array detector (DAD, Agilent Technologies, Palo Alto, CA). Separation of isoflavones was achieved using a reversed phase C₁₈Luna column (Phenomenex, Lorraine, CA, USA, 150×4.6 mm; particle size 5 µm), preceded by a guard column (Phenomenex, 4×3.0 mm) of the same stationary phase. The HPLC gradient and conditions were similar to those described by Lee et al. (2004) and Murphy et al. (1997) with minor modifications. Solvent A consisted of 1% (v/v) acetic acid in water and solvent B was 100% ace-

tonitrile. Flow rate was set to 0.6 ml/min and the column and the guard column were thermostatically controlled at 25 °C. For the first 5 min gradient was maintained at 15% B. The gradient elution was changed from 15% to 45% B in a linear fashion for a duration of 44 min. Column was washed at 100% B for 5 min and equilibrated for additional 15 min at 15% B. Total analysis time per sample was 70 min. HPLC chromatograms were detected using a photo diode array UV detector. The structures for all isoflavones were confirmed by comparison of retention time, UV spectra and mass spectral analysis. Peak areas were integrated for quantitation. As authentic purified standard of for all twelve isoflavones were not easily available. Comparison of extraction efficiencies was achieved by comparing HPLC peak areas.

2.6. Statistical analysis

All statistical analyses were conducted using ANOVA and *t*-test using a statistical analysis software (SAS[®] v 9.1.2 from SAS Institute, Inc., Cary, NC, USA). Individual peak areas and the total areas were compared with each other using least significant difference (LSD) method to determine any significant difference ($p < 0.001$) among them.

3. Results and discussion

3.1. Effect of solvent composition on extraction of isoflavones

A typical HPLC chromatogram of the isoflavones extracted from soybean sample is shown in Fig. 2. The structures of the 12 isoflavones were confirmed by comparison of the UV spectra, retention times as well as mass

spectral analysis as reported in literature (Griffith & Collison, 2001; Klejdus et al., 2004; Wu et al., 2004). The twelve isoflavones were identified as 1. daidzin, 2. glycitin, 3. genistin, 4. malonyldaidzin, 5. malonylglycitin, 6. acetyldaidzin, 7. acetylglycitin, 8. malonylgenistin, 9. daidzein, 10. glycitein, 11. acetylgenistin, and 12. genistein. Table 2 summarizes the HPLC peak areas of each of the twelve identified isoflavones with four different solvent mixtures commonly deployed for extraction of isoflavones as reported in the peer-reviewed literature (Table 1). We have evaluated extraction efficiencies of isoflavones from soybeans with three additional solvent mixtures (dimethyl sulphoxide:ethanol:water (5:70:25, v/v/v); dimethyl sulphoxide:acetonitrile:water (5:58:37, v/v/v); and Genapol:water (5:95, v/v)). Addition of dimethyl sulphoxide to the two previously optimized extraction solvent mixtures (ethanol:water (70:30, v/v); acetonitrile:water (58:42, v/v)) was carried out based on the solubility of isoflavones reported at the Sigma-Aldrich website (<http://www.sigma-aldrich.com>). In addition, Griffith and Collison (2001) reported improvement (0.7–10.6%) in the extraction efficiency of different isoflavones from soy samples extracted with internal standard apigenin dissolved in dimethyl sulphoxide. The authors suggested that marginal increase in isoflavones content might be attributed to the lack of acid or to the presence of small quantity of dimethyl sulphoxide. The authors suggested that additional research was needed to evaluate the influence of dimethyl sulphoxide on extraction efficiency of isoflavones.

In the current study, as purified standards for all 12 isoflavones were either not commercially available or very expensive, evaluation of extraction efficiency was carried out by comparison of HPLC areas. Individual peak areas and the HPLC profiles for different isoflavones were

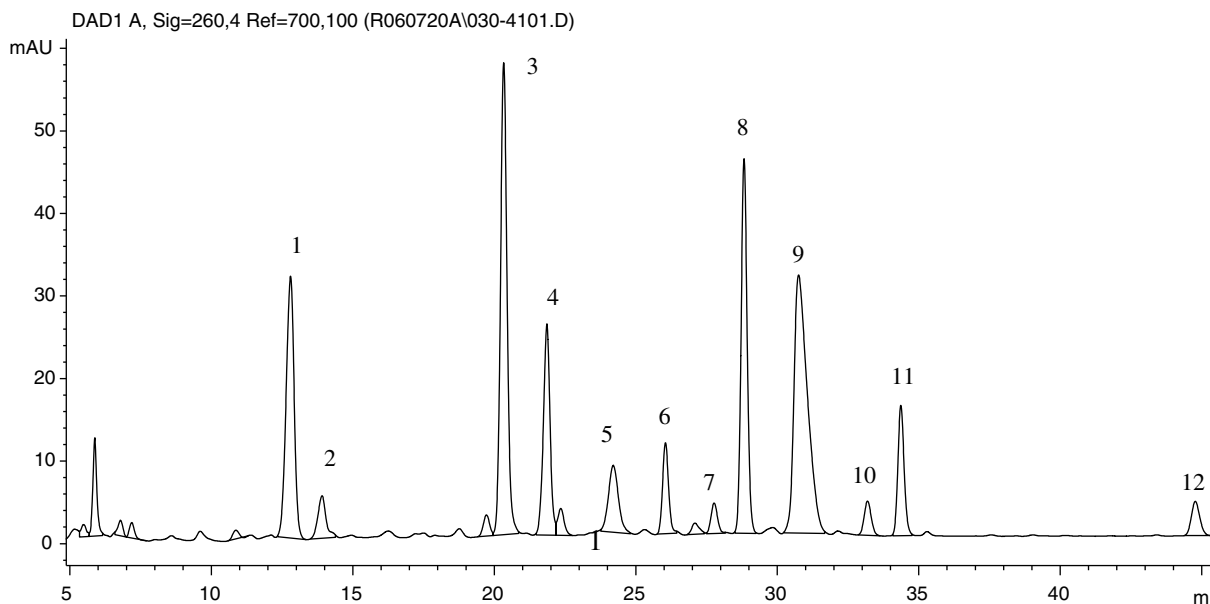


Fig. 2. A typical HPLC profile of isoflavones extracted from soybean. The peaks are identified as 1. Daidzin, 2. Glycitin, 3. Genistin, 4. Malonyldaidzin, 5. Malonylglycitin, 6. Acetyldaidzin, 7. Acetylglycitin, 8. Malonylgenistin, 9. Daidzein, 10. Glycitein, 11. Acetylgenistin, and 12. Genistein.

Table 2
Average HPLC peak areas from four replicate soybean extracts analyzed in duplicate for 12 isoflavones extracted with eight different solvents mixtures

Solvents	Areas												SUM
	Daidzin	Glycitin	Genistin	Malonyl Daidzin	Malonyl Glycitin	Acetyl Daidzin	Acetyl Glycitin	Malonyl Genistin	Daidzein	Glycitein	Acetyl Genistin	Genistein	
58% ACN	265.8d	62.0d	363.8e	174.6c	0.0c	46.8d	295.4d	32.5b,c	0.0c	51.7c	65.6d	46.5c	1401.8e
58% ACN, 5% DMSO	175.3f	53.0d	662.6d	351.2a	0.0c	157.2a	606.1b	30.5c	0.0c	73.2a,b	213.2a	84.0b	2406.4d
70% ETOH	571.7c	88.8c	784.4c	386.3a	226.6b	78.6c	58.1e	38.1b	752.8b	61.0b,c	67.6d	68.3b	3182.7c
70% ETOH, 5% DMSO	662.2b	111.2b	920.4b	382.6a	235.5a	117.2b	707.5a	69.2a	1054.4a	76.9a	172.8b	83.9b	4594.3a
90% MEOH	1065.1a	179.9a	1476.2a	243.8b	0.0c	109.5b	430.4c	0.00d	0.0c	83.1a	152.0c	106.0a	3846.2b
Water	243.9d,e	37.1e	309.2e,f	65.2d	0.0c	25.5e	95.4e	0.00d	0.0c	22.4d	25.8e	12.4d	837.1f
5% genipol	191.0e,f	31.5e	230.4f	42.3d	0.0c	21.4e	61.4e	0.00d	0.0c	15.2d	19.4e	15.5d	628.4g
LSD	68.2	11.2	86.3	58.4	7.6	15.8	63.3	7.0	6.5	12.2	13.2	17.9	128.4

The average areas of the peaks with any identical letter are not significantly different with each other at the $\alpha = 0.05$ level by least significant difference test; $n = 3$. ACN, acetonitrile; DMSO, dimethyl sulphoxide; ETOH, ethanol; MEOH, methanol; LSD, least significant difference.

Table 3
Comparison of the recovery of individual isoflavones extracted from soybeans using different solvent mixtures

Solvents	Areas (%)												SUM
	Daidzin	Glycitin	Genistin	Malonyl Daidzin	Malonyl Glycitin	Acetyl Daidzin	Acetyl Glycitin	Malonyl Genistin	Daidzein	Glycitein	Acetyl Genistin	Genistein	
58% ACN	25.0	34.5	24.6	45.2	0	29.77	41.8	47.0	0.0	67.2	30.8	43.9	30.5
58% ACN, 5% DMSO	16.5	29.5	44.9	100.0	0	100.0	85.7	44.1	0.0	95.2	100.0	79.2	52.4
70% ETOH	53.7	49.4	53.1	100.0	96.2	50.0	8.2	55.1	71.4	79.3	31.7	64.4	69.3
70% ETOH, 5% DMSO	62.2	61.8	62.3	100.0	100.0	74.6	100.0	100.0	100.0	100.0	81.1	79.2	100.0
90% MEOH	100.0	100.0	100.0	63.1	0	69.7	60.8	0.0	0.0	100.0	71.3	100.0	83.7
Water	22.9	20.6	20.9	16.9	0	16.2	13.5	0.0	0.0	29.1	12.1	11.7	18.2
5% genipol	17.9	17.5	15.6	11.0	0	13.6	8.7	0.0	0.0	19.8	9.1	14.6	13.7

ACN, acetonitrile; DMSO, dimethyl sulphoxide; ETOH, ethanol; MEOH, methanol.

significantly dissimilar with different extraction solvent mixtures. Optimum extraction of the total isoflavones content as measured by the HPLC peak areas was obtained when extractions were performed with dimethyl sulphoxide:ethanol:water (5:70:25, v/v/v) solvent mixture. The extraction efficiency of all other solvent mixtures were compared with optimized extraction solvent mixture (dimethyl sulphoxide:ethanol:water (5:70:25, v/v/v)). Lowest yields (13.7 and 18.2%) of total isoflavones were obtained with water and 5% Genapol in water. Evaluation with nonionic surfactant oligo (ethyleneglycol) monoalkyl ether (Genapol X-080) was carried out based on the recent study on extraction of isoflavones diadzein from *Puerariae radix* (He et al., 2005). The authors obtained optimum extraction of isoflavones with 5% Genapol X-080 (w/v). However, in the present study significant decrease in isoflavones yields were obtained with 5% Genapol in water. This variation in extraction efficiencies may be attributed to the differences in the sample matrix, isoflavones content and composition. Only 30.5% of the isoflavones were extracted with acetonitrile:water (58:42, v/v) solvent mixture. The yield of isoflavones content increased to 52.3%, when extractions were performed with dimethyl sulphoxide:acetonitrile:water (58:37:5, v/v/v) solvent mixture. Similar increase in extraction yields of isoflavones were obtained when 5% DMSO was added to ethanol:water (70:30, v/v) solvent mixture. Intermediate yields (83.7%) of total isoflavones were extracted with (methanol:water, 90:10, v/v).

There were significant differences ($p < 0.001$) in individual isoflavones content extracted from a soybean sample with different solvents mixtures (Table 3). Maximum yields of the three glycosylated isoflavones (daidzin, glycitin and genistin) were obtained with methanol:water (90:10, v/v). However, the optimum yields for all the other nine iso-

flavones (malonyldaidzin, malonylglycitin, acetyldaidzin, acetylglycitin, malonylgenistin, daidzein, glycitein, acetylgenistin, and genistein) were obtained with (dimethyl sulphoxide:ethanol:water (5:70:25, v/v/v)) (Fig. 3). All twelve isoflavones were detected in measurable quantities only with two different extraction solvent mixtures (dimethyl sulphoxide:ethanol:water (5:70:25, v/v/v) and ethanol:water (70:30, v/v)). The two isoflavones, daidzein and malonylglycitin were not extracted in measurable amounts with five other extraction solvent mixtures acetonitrile:water (58:42, v/v); dimethyl sulphoxide:acetonitrile:water (5:58:37, v/v/v); methanol:water (90:10, v/v); water; and Genapol:water (5:95, v/v) evaluated in the current study. Similarly, Klejdus et al. (2005) extracted trace quantity of daidzein (1.2% of total isoflavones extracted) and did not detect presence of any malonyl glycitin from soybeans with methanol:water (90:10, % v/v) as extraction solvent. In another study, Lin and Giusti (2005) reported trace quantities (1.2% and 1.3%) of the two isoflavones daidzein and malonylglycitin with acetonitrile:water (58:42, v/v) solvent mixture. Insignificant amounts of the above two isoflavones (daidzein and malonyl-glycitin) were extracted from soybeans with superheated water at elevated pressures (Li-Hsun, Ya-Chuan, & Chieh-Ming, 2004).

3.2. Effect of extraction techniques on assay of isoflavones from soybeans

Various extraction techniques have been used by different research groups for extraction of isoflavones from soybeans (Table 1). Comparison of extraction of isoflavones with single optimized extraction solvent mixture (dimethyl sulphoxide:acetonitrile:water, 5:58:37, v/v/v) with same solid-to-solvent ratio was carried out using six commonly used extraction techniques (PLE, sonication, Soxhlet,

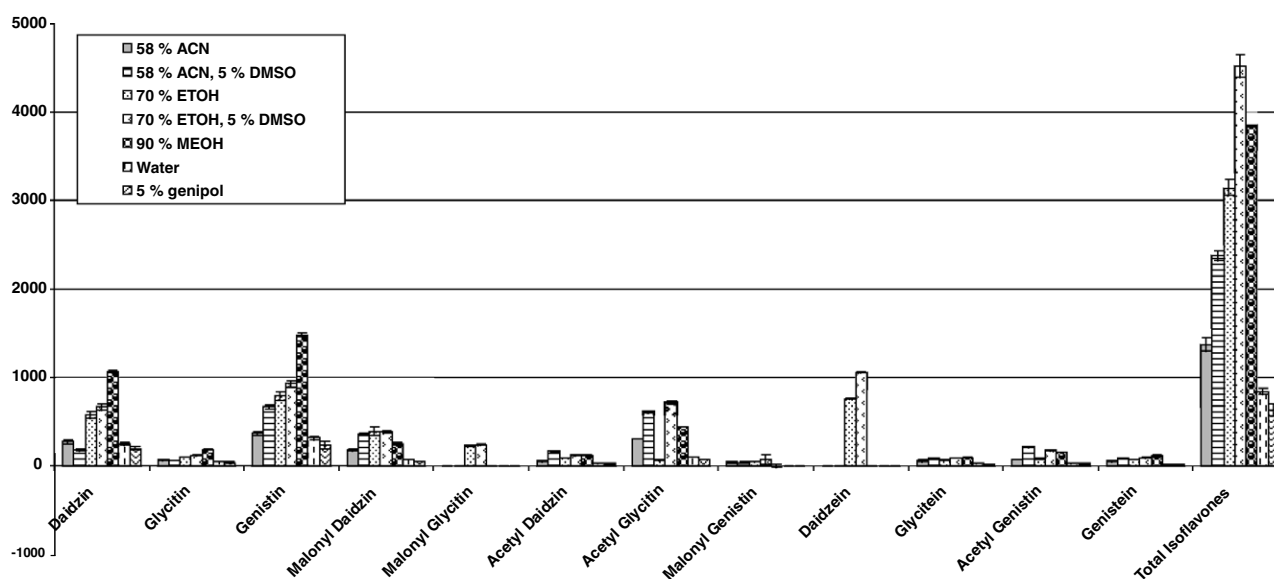


Fig. 3. Influence of extraction solvent composition of assay of 12 isoflavones from soybeans.

Table 4

Isoflavone peak areas obtained by extraction with different methods such as PLE, sonication, Soxhlet, shaker, vortex, and stirring using 70% ETOH, 5% DMSO from Organic soybeans

Extraction method	Areas												
	Daidzin	Glycitin	Genistin	Malonyl Daidzin	Malonyl Glycitin	Acetyl Daidzin	Acetyl Glycitin	Malonyl Genistin	Daidzein	Glycitein	Acetyl Genistin	Genistein	SUM
PLE	662.3b	111.2b	920.5b	382.7c	235.5a	117.2a	69.2a	707.5c	1054.5a	76.9b,c	172.8a	83.9b	4594.3a
Sonication	426.7d	87.6c	648.8d	676.8b	213.5a	14.7c	6.96c	969.1b	1030.8b	95.5a	30.0c	83.7b	4284.1b
Soxhlet	804.1a	124.5a	1133.2a	302.1d	0.0c	61.1b	0.0d	460.9d	0.0c	73.1c	94.2b	85.5b	3138.7c
Shaker	478.4c	82.7c	688.6c	715.5a	0.0c	0.0d	35.9b	1076.5a	0.0c	76.1b,c	0.0d	85.1b	3238.7c
Vortex	451.2c,d	76.9d	655.1d	679.3b	0.0c	0.0d	32.0b	958.7b	0.0c	74.6b,c	0.0d	83.6b	3011.5d
Stirring	470.5c	74.1d	690.7c	728.6a	0.0c	0.0d	31.5b	1048.0a	0.0c	79.2b	0.0d	90.6a	3213.2c
LSD	34.8	5.3	33.1	21.8	5.9	5.0	6.9	64.3	3.0	5.6	6.0	4.2	121.8

The average areas of the peaks with any identical letter are not significantly different with each other at the $\alpha = 0.05$ level by least significant difference test; $n = 3$. PLE, accelerated solvent extraction LSD, least significant difference.

shaker, vortex, and stirring). Table 4 shows the result of individual and total isoflavones content isolated by six different extraction procedures. The results indicate that optimum yields of total isoflavones were extracted by PLE procedure. Only marginal decrease (6.7%) in yields of isoflavones content was obtained when extractions were performed with sonication. The extraction efficiency of the other four procedures (stirring, Soxhlet, shaker and vortex) was between 65.6% and 70.4% as compared to PLE. Rostagno, Araujo, and Sandi (2002) had compared the extraction efficiencies of soybean isoflavones by three extraction techniques (sonication, Soxhlet, and supercritical fluid extraction). The authors reported that optimum extractions were obtained with sonication procedure. The extraction efficiency for Soxhlet and supercritical fluid extraction were 68.3% and 27.7% respectively as compared to the sonication procedure. Similar extraction recoveries for total isoflavones were obtained with Soxhlet procedure (73.2%) as compared to sonication in the present study. However, in another study, Delmonte, Perry, and Rader (2006) reported similar recoveries for total isoflavones by sonication and shaker techniques. These results are different from our present study as we observed that extraction efficiencies with total isoflavones by shaker procedure were only 75.6% efficient as compared to sonication procedure. This variation in isoflavones extraction yields from soybeans by Delmonte et al. (2006) may be attributed to differences in extraction solvents, solid-to-solvent ratios, and temperature used during extraction by shaker and sonication procedures. However in the present study, same extraction solvent mixture, same solid-to-solvent ratio and same extraction conditions were used for sonication and shaker procedures.

4. Conclusion

This study as well as other previously reported publications (Luthria & Mukhopadhyay, 2006; Luthria, Mukhopadhyay, & Kwansa, 2006; Mitra & Brukh, 2003; Mukhopadhyay, Luthria, & Robbins, 2006) and reviews (Antolovich, Prenzler, Robards, & Ryan, 2000; Escarpa

& Gonzalez, 2001; Luthria, 2006; Nacz & Shahidi, 2004) clearly illustrates that it is essential to systematically evaluate and optimize the extraction solvent composition, extraction procedure and conditions for accurate and reproducible estimation of structurally diverse phenolic compounds from different food matrices. In the present study, optimum isoflavones recoveries from soybean samples were obtained with dimethyl sulphoxide:ethanol:water (5:70:25, v/v/v) solvent mixture using a PLE. Several thousands different phenolics with wide range of polarities, forms (aglycon, glycosylated, acetylated/malonylated, esterified to acids, etc.) have been reported in literature. In addition, phenolic compounds may be associated with different cellular components such as cell walls, proteins, lipids etc. It is critical to optimize sample preparation procedures for accurate analysis of different classes of phenolic compounds isolated from various food matrices.

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