Influence of Sample Preparation on the Assay of Isoflavones

Authors **Devanand L. Luthria**[1], **Savithiry S. Natarajan**[2]

Affiliations
[1] Food Composition and Methods Development Laboratory, Beltsville Human Nutrition Research Center, ARS, USDA, Beltsville, Maryland, USA
[2] Soybean Genomics and Improvement Laboratory, Plant Sciences Institute, ARS, USDA, Beltsville, Maryland, USA

Key words
- isoflavones
- extraction solvents composition
- extraction techniques and conditions
- sample preparation
- foods and plants matrices

Abstract

The complexity of sample matrices, coexistence of multiple forms of bioactive phytochemicals, and their interaction of with other cellular components pose a significant challenge for optimized extraction and accurate estimation of bioactive phytochemicals in foods and dietary supplements. This article discusses the significance of optimizing extraction procedures for accurate assay of phytochemicals from different matrices using bioactive isoflavones as model substrate because isoflavones are known to exist in nature as free aglycones or as conjugates with sugars and/or acids. The wide structural diversity and polarities of free and conjugated isoflavones makes optimum extraction and accurate quantification of isoflavones a challenging task. This paper reviews variables, extraction solvent composition (aqueous-organic solvents mixtures at different acidification levels), physical extraction techniques (Soxhlet, stirring, ultrasonic, microwave, pressurized, supercritical-fluid, high-speed counter-current chromatography), and parameters (temperature, pressure, number of cycles, solid-solvent ratio) that influence quantitative extraction of isoflavones from different matrices. In addition, this review covers a brief overview of structures, sources, bioactivities, separation, and detection used for isoflavones analysis. Optimum extraction efficiencies of isoflavones were obtained with EtOH : H_2O : DMSO (70 : 25 : 5, v/v/v) as the extraction solvent and acidification of extraction solvent favored partial degradation of conjugated forms to their corresponding aglycones. Accurate quantification of isoflavones in foods, plants, and dietary supplements will allow researchers and regulators to provide more precise guidelines on dietary intake and safety levels necessary to achieve optimum health.

received October 7, 2008
revised January 12, 2009
accepted January 21, 2009

Bibliography
DOI 10.1055/s-0029-1185439
Published online March 13, 2009
Planta Med 2009; 75: 704–710

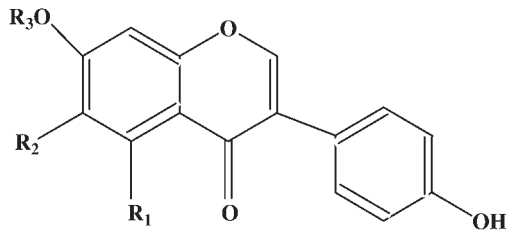
ISSN 0032-0943

Correspondence
Devanand Luthria
Food Composition and Methods Development Laboratory
Room 202 B, Building 161, BARC-E
Beltsville Human Nutrition Research Center
USDA
10300 Baltimore Ave.
Beltsville
MD 20705-3000
USA
Phone: + 1 301 504 72 47
Fax: + 1 301 504 83 14
D.Luthria@ars.usda.gov

Introduction

The importance of bioactive phytochemicals in human health and nutrition is well documented in peer-reviewed scientific literature [1–3]. A wide range of plant products in their natural and processed forms are used for the treatment of a multitude of diseases [4–6]. The procedure for the assay of bioactive phytochemicals from plant matrices generally comprises of four steps: 1) sample preparation, 2) analytical separation, 3) detection, and 4) data collection and processing [7]. Significant advancements have been made in the final three steps; however only limited progress has been made in the first sample preparation step. Sample preparation is often considered as a rate-limiting step and roughly accounts for approximately 30% of the analytical error [8]. This sample preparation step is often not well optimized and frequently considered as a means to an end by a large number of analysts [7,8]. Sample preparation in itself encompasses multiple steps such as grinding, sieving, extraction, preconcentration, filtration, and derivatization.

There have been excellent reviews on analytical separation and analysis of flavonoids in recent years [9–12]. This review focuses on the influence of sample preparation on extraction of bioactive isoflavones from different matrices. It depicts the wide variation in the methodologies used for extracting isoflavones by different research groups. Isoflavones were selected as a model substrate because they exhibit a wide range in polarities and they are known to exist in multiple forms, aglycones as well as in conjugated forms with sugars and acids. Determination of the accurate concentration of total isoflavones content in plants and food matrices is hindered as purified standards of multiple forms of isoflavones are either unavailable or very expensive. In addition, a brief



Name	R_1	R_2	R_3
Daidzein	H	H	H
Glycitein	H	OCH_3	H
Genistein	OH	H	H
Daidzin	H	H	Glucosyl
Glycitin	H	OCH_3	Glucosyl
Genistin	OH	H	Glucosyl
Acetyl or Malonyl daidzin	H	H	Glucosyl-$COCH_3$ or Glucosyl-$COCH_2COOH$
Acetyl or malonyl glycitin	H	OCH_3	Glucosyl-$COCH_3$ or Glucosyl-$COCH_2COOH$
Acetyl or malonyl genistin	OH	H	Glucosyl-$COCH_3$ or Glucosyl-$COCH_2COOH$

Fig. 1 Chemical structures of different isoflavones extracted and identified from soybeans extract.

discussion on different sources, bioactivities, biosynthesis, and HPLC separation has also been included to provide an overview of current ongoing research in the area of isoflavones.

Isoflavones: Structure, Sources, and Bioactivities

Isoflavones are diphenolic phytochemicals that naturally occur in members of the legume family Fabaceae [13]. Isoflavones are oxygen heterocycles containing a 3-phenylchroman skeleton that is hydroxylated at 4′ and 7 positions [14] (**Fig. 1**). In isoflavones the B ring is substituted on the third carbon atom of the pyrone ring as compared to position 2 in flavones. Isoflavones are biosynthesized from the general phenylpropanoid pathway which produces all flavonoid compounds in higher plants [15].

Soybean is the best known natural source of isoflavones [16]. Other natural sources of isoflavones are chickpeas, various beans and sprouts, red clover, fruits (currants, raisins, prunes, cranberries, yellow honeydew, osage orange, strawberries), nuts (hazelnut, chestnut, coconut, peanut), sesame seeds, vegetables (asparagus, potatoe, aubergine, broccoli sprout, corn, turnip, tomato), and cereals [17–21]. Isoflavones commonly exist in free (aglycone) and conjugated forms (acetyl, malonyl, glycosylated). Soy and red clover are the two richest natural sources of isoflavones. In both the isoflavones are predominantly present as glycosides and malonylated glycoside conjugates such as 7-*O*-β-D-glucoside and 6″-*O*-malonylglucoside of daidzein and genistein in soy and 7-*O*-β-D-glucoside and 6″-O-malonylglucoside of formononetin and biochanin A in red clover. Based on the substitution pattern of carbons 5 and 6, three aglycone forms of isoflavones commonly found in soybeans are daidzein, genistein, and glycitein. The structures of some free and conjugated forms of isoflavones are presented in **Fig. 1**.

Isoflavones are chemically similar in structure to the female hormone estrogen [21] and are believed to provide protection against osteoporosis, cardiovascular disease, and certain types of cancers [22–27]. There are several reports suggesting that regular consumption of isoflavones reduces the incidence of hot flashes and other menopausal symptoms [5,25–27]. In Japan, where isoflavones consumption is higher than in many other countries in the West, the incidences of many of the diseases listed above are much lower. A recent review by Branca and Lorenzetti [28] suggested that consumption of 50–110 mg of isoflavones aglycon per day for 6–12 months improves mineral bone density.

Isoflavones Extraction and Analysis

The data presented in **Table 1** illustrate the wide range of solvent mixtures and physical techniques that have been published in peer-reviewed journals for the extraction of isoflavones from different plant matrices. The most frequent techniques used for extraction include, Soxhlet extraction, ultrasonic-assisted extraction, pressurized liquid extraction (PLE), microwave assisted extraction (MAE), supercritical fluid extraction (SFE), stirring, and mechanical shaking. The extraction time used by different researchers varied between 20 min and 6 hours. In most soybean publications, extractions have been carried out directly without defatting. However a few researchers have defatted soybean prior to isoflavone extraction. The choice of extraction solvents varied drastically, including a wide range of aqueous dilutions of acetonitrile (ACN), ethanol (EtOH), and methanol (MeOH) with varying acidity.

Table 1 Summary of the recent procedures used for the extraction of isoflavones from different plant matrices.

Serial #	Extraction procedure	Best Solvent	Pub. year	Reference
1	2 g 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	[54]
2	Freeze-dried soybeans were extracted with pressurized liquid extractor (PLE) with different proportions of (30–80%) aqueous EtOH and MeOH solvent mixtures at different temperatures, pressures, solid-to-solvent ratios. Optimizations of PLE conditions are described.	70% EtOH	2002	[55]
3	Soy meal was extracted with acidified ACN (84%) by shaking sample for 2 h at room temperature.	84% ACN	2004	[56]
4	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	[27]
5	The authors developed a process for extracting and purifying isoflavones from defatted soybean flakes using superheated water at elevated pressures using simulation optimization approach known as "steepest ascent". An experimental design was adopted to optimize extraction conditions for isoflavones. The optimum conditions were determined as 110 °C and 641 psig (4520 kPa) over 2.3 h of extraction.	superheated water	2004	[57]
6	A non-ionic surfactant oligo(ethylene glycol) monoalkyl ether (Genapol X-080) was used for the extraction of daidzein from Puerariae Radix.	5% Genapol (X-080, w/v)	2005	[30]
7	Comparison of different extraction conditions (extraction time, temperature, pressure, cycles, solvents) and techniques (PLE, PLE + sonication, sonication, and Soxhlet).	90% MeOH	2004, 2005	[58, 59]
8	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	[29]
9	Defatted soy samples were extracted with 80% ACN, 80% MeOH and 80% EtOH by stirring and sonicating samples. The authors suggest replicate extractions are essential for optimum extraction. Significant decrease in isoflavones yields were obtained with one extraction cycle.	similar yields with all 3 solvents	2005	[60]
10	The authors used ultrasound-assisted extraction procedure. The extraction protocol consisted of extracting 0.25 g of the freeze-dried sample with 25 mL of 50% EtOH at 60 °C in an ultrasonic bath for 20 min.	50% EtOH	2007	[35]
11	The authors used *n*-butanol/water, two phase solvent system for the extraction of isoflavones from the stem of *Pueraria lobata* (Willd.) followed by alumina column purification and recrystallization.	1 : 1 (v/v) *n*-butanol : water	2007	[41]
12	The authors systematically compared three 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) and identified increased extraction efficiencies with dimethyl sulfoxide : ethanol : water (5 : 70 : 25, % v/v/v). In addition, authors carried out comparison between six commonly used extraction techniques [shaking, vortexing, sonication, stirring, Soxhlet and pressurized liquid extraction (PLE)] and found that PLE provided optimum efficiency recoveries.	dimethyl sulfoxide: ethanol : water (5 : 70 : 25, %v/v/v)	2007	[34]
13	The authors compared extraction yields of isoflavones from soybean cake by solvent and supercritical carbon dioxide and studied the conversion of isoflavone glucosides to the biologically active aglycone by employing β-glucosidase.	supercritical carbon dioxide	2008	[42]

Influence of physical techniques and extraction conditions (solvent composition, surfactant addition, solid-to-solvent ratio, time, particle size, and temperature) on assays of isoflavones

Lin and Giusti described the influence of solvent polarity and acidity on extraction efficiency of isoflavones from soybeans [29]. The authors carried out extractions with six different solvent systems that were a combination of three polarities and two acidity levels (83% ACN, 83% acidified ACN, 80% MeOH, 80% acidified MeOH, 58% ACN, and 58% acidified ACN). Acidified solvents were prepared by mixing 2 mL of 0.1 N HCl with 12 mL of solvent. The authors showed that solvents of high polarity (Snyder polarity index, P′ = 6.7–7.4) provided higher yields of isoflavones extracted from soybeans. Acidification of the solvent favored transformation of conjugated isoflavones to their respective aglycones. The use of acidic solvents should be avoided if the purpose of the study is the quantification and evaluation of bioactivity and bioavailability of different forms of conjugated isoflavones. Among the six solvent systems investigated in their study, the authors observed that 58% acetonitrile without acidification provided the highest yield of isoflavones without modification of their conjugated structures.

He et al. used a non-ionic surfactant oligo(ethylene glycol) monoalkyl ether (Genapol X-080) for the extraction of daidzein from Puerariae Radix, a traditional Chinese herb used as an antimicrobial, pain reducing, and appetite-inducing agent [30]. The optimization of extraction of daidzein from Puerariae Radix was carried out using an ultrasonic extraction technique. The authors showed that micelle-mediated extraction with a non-ionic surfactant could be beneficial for solubilization, purification, and preconcentration of active ingredients from herbal supplements. The authors compared the influence of eight different solvents [5% Genapol, methanol, acetic ether, ethanol, 50% methanol, dichlormethane : methanol (1 : 4, v/v), cyclohexane and *n*-hexane] on extraction efficiency of daidzein from Puerariae Radix. In addition, they also investigated the influence of the liquid-to-solid ratio, time, and physical techniques (ultrasonic extraction versus shake flask extraction) on isoflavone extractions. Optimum extraction of daidzein was obtained with 5% Genapol X-080 with 25 : 1 (mL/g) liquid-to-solid ratio using ultrasonic assisted extraction for 45 min.

Recently, Lee and Lin compared ultrasonic and pressurized solvent extraction for the determination of three isoflavones (puerarin, daidzin, and daidzein) in Puerariae Radix [31]. The powdered sample was mixed with 95% ethanol using a magnetic stirrer for 5 min. The slurry was subjected to ultrasonic irradiation at different powers 20, 40, 60, and 80 MJ. The authors also examined extraction efficiencies as a function of the particle size of the sample and determined that extraction of isoflavones increased with decreasing particle size. A better yield of isoflavones was obtained with the ultrasonic method as compared to PLE. The extraction conditions for PLE were: solvent: 95% EtOH; extraction volume: 10 mL; temperature: 60, 80, 100 °C; static time: 10 min; purge time: 60 s; static cycle: 1; pressure 1400 psi. The main drawback of this study was that different mean particle sizes (135–158 µm for PLE and 12–33 µm for ultrasonic-assisted extraction) were used for extraction. It is well documented in the literature that particle size influences extraction efficiency [32,33].

Luthria et al. examined the influence of solvent composition and physical techniques on the extraction of isoflavones from soybeans [34]. This detailed systematic study was undertaken to investigate the wide variations in extraction solvents and techniques reported in peer-reviewed publications (**Table 1**). They compared extraction of eight different solvent mixtures [ACN : H_2O, 58 : 42, v/v; ACN : H_2O : DMSO, 58 : 37 : 5, v/v/v; EtOH : H_2O, 70 : 30, v/v; EtOH : H_2O : DMSO, 70 : 25 : 5, v/v/v; MeOH : H_2O, 90 : 10, v/v; H_2O, and 5% Genapol X-080 (w/v)] for extraction of isoflavones from ground soybeans using a PLE technique with the following parameters: extraction cell volume (11 mL); temperature 100 °C; static time 7 min; flush volume 75%. Optimum extraction efficiencies of isoflavones were obtained with EtOH : H_2O : DMSO (70 : 25 : 5, v/v/v) as the extraction solvent. Only 83.7% of the total isoflavones were extracted with MeOH : H_2O (90 : 10, v/v). Intermediate extraction yields (30.5% to 69.3%) of isoflavones were obtained with other three solvents (ACN : H_2O, 58 : 42, v/v; ACN : H_2O : DMSO, 58 : 37 : 5, v/v/v; EtOH : H_2O, 70 : 30, v/v) and the lowest yields (13.6–18.2%) were obtained with 100% water and 5% Genapol X-080.

Rostagno et al. developed a rapid method for the assay of isoflavones from soy beverages blended with fruit juices [35]. The authors evaluated the influence of solvents (MeOH and EtOH), sample-to-solvent ratio (5 : 1 to 0.2 : 1), temperature (10–60 °C), and extraction time (5–30 min). The optimized procedure consisted of extracting samples with ethanol in ultrasonic mixing at 45 °C for the duration of 20 min with a sample-to-solvent ratio of 0.2 : 1 (v/v). The same group also optimized MAE of isoflavones using a microwave extractor ETHOS 1600. Extractions were performed in a batch at 500 W using magnetic stirring at 50% of nominal power [36]. The authors compared extraction efficiencies of isoflavones with different solvents (MeOH, EtOH, 30–70% in water and water), temperature (50–150 °C), sample size (0.1–1.0 g), and time (5–30 min). The optimum extraction conditions were obtained with 0.5 g of sample using 50% EtOH as extraction solvent with extraction time and temperature of 20 min and 50 °C, respectively.

Application of solid-phase extraction (SPE) for enrichment or purification of isoflavones

SPE is a rapid, cost-efficient sample preparation technique used either for partial purification or enrichment of analyte concentration. This technique usually eliminates interferences and undesirable matrix components. SPE has mainly replaced classical liquid-liquid extractions which require large quantities of organic solvents and are not environmentally friendly. In SPE, solids are partitioned between a mobile phase and a stationary phase that has a greater affinity for the analytes of interest. SPE cartridges with several different stationary phases are commercially available. Rostagno et al. developed an SPE procedure for concentration and clean-up of soy isoflavones extract [37]. The authors compared eight different SPE cartridges from various suppliers (Bond Elut C-18, Varian; Bond Elut ENV, Varian; Discovery DSC-18, Supelco; Strata C-18E, Phenomenex; Strata SDB-L, Phenomenex; Strata X, Phenomenex; LiChorlut EN, Merck; Oasis HLB, Waters) containing a wide spectrum of sorbents (octadecyl silica, styrene-divinylbenzene, modified divinylbenzene, ethylvinylbenzene and styrene divinylbenzene, and divinyl-benzene-co-*N*-vinylpyrrolidone). Optimum retention and recovery was achieved with the Strata X cartridge (modified divinylbenzene, 200 mg solid phase) procured from Phenomenex.

Utilization of high speed countercurrent chromatography (HSCCC) for isoflavones extraction

HSCCC is developed from countercurrent chromatography (CCC), a separation technique based on liquid-liquid partition has also been used for isolation and purification of isoflavones from different plant matrices [38–40]. Compared to conventional chromatographic separation, irreversible absorptive loss on to solid support is prevented by the use of HSCCC. Isoflavones purification using HSCCC was achieved using two-phase solvent system comprising of hexane : ethyl acetate : *n*-butanol : methanol : acetic acid : water in 1 : 2 : 1 : 1 : 5 : 1 (v/v) proportion by Du et al. [38]. These authors were able to separate isoflavones with over 90% purity from soybean. In another study, Cao et al. utilized HSCCC for the separation and purification of puerarin and related isoflavones from the crude extract of *Pueraria lobata* using ethyl acetate : *n*-butanol : water (2 : 1 : 3, v/v) [39]. In a recent study, Ye et al. carried out preparative isolation and purification of three roteinoids and a single 4′,5′-dimethoxy-6,6-dimethyl pyranoisoflavone was achieved using a biphasic solvent system composed of *n*-hexane : ethyl acetate : methanol : water (1 : 0.8 : 1 : 0.6, v/v) from the seeds of *Millenttia pachycarpa* [40].

Xu and He developed a new process for the extraction of isoflavones from the stems of *Pueraria lobata* using a two-phase solvent system (*n*-butanol : water), alumina column purification, and recrystallization [41]. The authors evaluated the effect of solvent type and determined that a two-phase solvent system [*n*-butanol : water (1 : 1, v/v)] provided maximum yields of diadzein as well as total isoflavones, compared to the common single-phase extraction solvents. The process developed by the authors is economically feasible and can be utilized for commercial scale extraction of isoflavones and daidzein.

Application of supercritical fluid extraction for analysis of isoflavones

Kao et al. compared the extraction of isoflavones from soybean cake with ethanol : water (1 : 1, v/v) and supercritical carbon dioxide with 1.6 mL of ethanol : water (70 : 30, v/v) as modifier [42]. The authors observed that maximum yield of malonyldaidzin, malonylglycitin, and malonylgenistin were generated at 60 °C and 350 bar. However, when the extractions were performed at 80 °C with 350 bar pressure, the yield of acetyl glucosides (acetyldaidzin, acetylglycitin, and acetylgenistin) and aglycones (daidzein, glycitein, and genistein) was significantly increased. The yields of the total isoflavones obtained with supercritical carbon

dioxide were lower than those for low pressure solvent procedures. In another recently published study, Zuo et al. evaluated the influence of modifier composition in terms of methanol content in water, particle size, temperature, pressure, CO_2 flow rate on isoflavones recovery from soybean [43]. The optimum isoflavones yield was obtained at 40 °C with 500 bar pressure using CO_2 flow rate of 9.8 kg/h containing a modifier 7.8 mass % of 80 % (v/v) aqueous methanol.

Extraction of isoflavones from culture media and biological fluids

Ksycinska et al. developed a method for the determination of genistein and its four newly identified derivatives in culture media using liquid-liquid extraction [44]. The extraction procedure involved centrifugation of the cell suspension followed by transfer of 0.5 mL of the supernatant in a 10-mL of glass tube containing either 3 mL of *tert*-butyl methyl ether or 4 mL of hexane:*n*-propanol (90 : 10, v/v) or 4 mL of *n*-hexane:*n*-propanol : acetic acid (95 : 5 : 1, v/v/v). The mixtures were shaken (10 min) and centrifuged for 10 min. After freezing the tube at − 70 °C for 15 min, the organic layer was transferred to glass tube and evaporated to dryness at 45 °C. The residue was redissolved in the mobile phase, filtered, and analyzed by HPLC.

A high throughput quantification of isoflavones in urine and serum samples using liquid chromatography/tendem mass spectrometry was recently described by Grace et al. [45]. This method used sample extraction and analysis in 96-well plates. Initially the biological samples are hydrolyzed with purified β-glucoronidase/aryl sulfatase from *Helix pomatia*. Aglycones were extracted from the hydrolysate via solid phase extraction on 96-well Strata-X plates using a robotic liquid handling system. Prior to SPE, methanol was added to each sample and the plates were conditioned with methanol followed by 30 % methanol before loading samples on to the plate. The plates were washed with 40 % methanol before eluting aglycones with acetonitrile : methanol (1 : 1, v/v). The analysis time was reduced to 10 min using column switching. This methodology is well suited for assaying large number of samples.

Separation and detection of isoflavones

The HPLC separation of different classes of isoflavonoids from soybeans samples was presented in a recent publication by Lin and Giusti [29]. The reverse phase HPLC separation was carried out on a C_{18} column with acidified aqueous acetonitrile as the mobile phase using a gradient elution. Isoflavones have characteristic UV absorption spectrum with two absorption maxima in the ranges 245 to 275 nm and 300 to 330 nm. The second band at the higher wavelength has a weaker relative intensity [21]. The identification of soy isoflavone aglycones and their conjugates (glycosides, glycoside acetylates and glycosides malonates) along with their MS fragmentations are discussed in detail in recent publications by Wu et al. [21], Otieno et al. [46], and Maul et al. [47]. A review on identification and quantification of isoflavones in biological fluids was published by Wilkinson et al. [48]. The authors compared the methods described in the literature and discussed the advantages and disadvantages associated with each method. The authors suggested the following order of sensitivity for the assay of isoflavones: radioimmunoassay > liquid chromatography-mass spectrometry (LC-MS) = HPLC-multichannel electrochemical detection (Coularray) > GC-MS-single ion monitoring > HPLC-ultraviolet (UV) diode array > HPLC-single channel electrochemical detection (ED). HPLC and LC-MS are the two most commonly utilized methods but the best sensitivity was reported with radioimmunoassay (0.002 pmol). In another review, Wu et al. discussed separation and detection by different procedures such as gas chromatography, HPLC, and capillary electrophoresis (CE) coupled with various detection methods such as UV, ED, fluorescence, mass spectrometry (MS), and nuclear magnetic resonance spectrometry (NMR) and non-chromatographic immunoassay methods [21]. Similar reviews of different detection methods for isoflavones were also published by Grynkiewicz et al. [49], and Vacek et al. [50]. A sensitive method for simultaneous quantification of puerarin and daidzein in rat plasma using HPLC with post-column modification and fluorescence detection was recently published by Zhai et al. [51]. This method required a linear gradient of 0.5 % aqueous acetic acid and 0.5 % acetic acid in acetonitrile. Addition of acetic acid allowed better separation but resulted in suppression of fluorescence signal as puerarin and daidzein have native fluorescence at pH 8.0 and 9.0. Improved sensitivity by over 500-fold for both analytes was obtained with post-column modification with alkaline (pH 9.0) ammonium acetate buffer. In a separate study, Careri et al. optimized extraction of isoflavone aglycones by 15 min ultrasonic irradiation of 0.1 g of finally ground soybean mixed with 2 mL acetoniltrile : water (80 : 20, v/v). The mixture was then subjected to microwave-assisted acidic hydrolysis at 600 W by adding 1 mL of 12 M HCl [52]. In another current publication Lv et al. discuss a single-step method for the purification of puerarin from Radix Puerariae using an *n*-octylamine-modified poly(methacrylate-co-dimethacrylate) monolith column. This column has potential for preparative-scale purification of puerarin as 12 mg per 2 mL loading resulted in 95 % purity with a recovery yield of 69 % [53].

Conclusions

The results summarized in this review illustrate the significance of different extraction parameters (physical technique, solvent composition, temperature, particle size, pressure, solid-to-solvent ratio, and number of extraction cycles) on the quantitative extraction of isoflavones from different food matrices. Extraction efficiences increased with the addition of small volumes of dimethyl sulfoxide and optimum yields of isoflavones were obtained with EtOH : H_2O : DMSO (70 : 25 : 5, v/v/v) as the extraction solvent. Acidification of extraction solvent favored partial degradation of conjugated forms to their corresponding aglycones. In addition, understanding acid and/or base treatments during sample preparation will influence conjugation patterns of isoflavones, which in turn impacts their solubility, bioavailability, and bioactivity. Utilization of green solvents as well as optimization of extraction procedures will significantly reduce chemical waste generated during extraction, thereby decreasing the operation and production costs. The process for optimization of an extraction procedure will depend on the type of analyte, its chemical form, the goal of the research project, and the composition of the sample matrix. This mini-review also illustrates that sample preparation methodology plays a vital role in accurate quantification. Accurate quantification of isoflavones and other bioactive compounds in foods, dietary supplements, and botanicals will allow researchers to accurately evaluate their role as it relates to human health and nutrition. Thus sample preparation that is often overlooked or considered as a trivial step and poorly described in the materials and methods section plays a vital role in accurately evaluating the role of phytochemicals. It is essential for

researchers, authors, and the editorial board members to check if the details regarding sample preparation procedures are properly documented in order to reproduce results published in peer-reviewed journals.

Acknowledgements

The authors would like to thank Sar Khader, Ronita Biswas, and Sudarsan Mukhopadhyay for their help in carrying out the research and preparation of this publication. We would also like to thank Mr. Bruce Richter from Dionex Corporation for providing supplies for ASE extraction. This research was supported by the Agricultural Research Service of the U.S. Department of Agriculture.

References

1 *Habauzit V, Horcajada M–N.* Phenolic phytochemicals and bone. Phytochem Rev 2008; 7: 313–344
2 *Chen L, Vigneault C, Vijaya Raghavan GS, Kubow S.* Importance of the phytochemical contents of fruits and vegetables to human health. Stewart Postharvest Rev 2007; 3: 1–5
3 *Hasler CM.* Functional foods: their role in disease prevention and health promotion. Food Technol 1998; 52: 63–70
4 *Mark-Herbert C.* Innovation of a new product category-functional foods. Technovation 2004; 24: 713–719
5 *Wansink B, Westgren RE, Cheney MW.* Hierarchy of nutritional knowledge that relates to consumption of functional foods. Nutrition 2005; 21: 264–268
6 *Wildman REC.* Nutraceuticals: a brief review of historical and teleological aspects. In: Wildman REC, editor. Handbook of nutraceuticals and functional foods. New York: CRC Press; 2001: 1–12
7 *Luthria DL.* Significance of sample preparation in developing analytical methodologies for accurate estimation of bioactive compounds in functional foods. J Sci Food Agric 2006; 86: 2266–2272
8 *Majors RE.* An overview of sample preparation methods for solids. LC-GC 1999; 17: S8–S13
9 *Escarpa A, Gonzalez MC.* An overview of analytical chemistry of phenolic compounds in foods. Critical Rev Anal Chem 2001; 31: 57–139
10 *Kaczk M, Shahidi F.* Extraction and analysis of phenolics in food. J Chromatogr A 2004; 1054: 95–111
11 *Rijke E, Out P, Niessen WMA, Ariese F, Gooijer C, Brinkman UAT.* Analytical separation and detection methods for flavonoids. J Chromatogr A 2006; 1112: 31–63
12 *Stalikas CD.* Extraction, separation, and detection methods for phenolic acids and flavonoids. J Sep Sci 2007; 30: 3268–3295
13 *Toebes AHW, Boer VD, Verkleij JAC, Lingeman H, Ernst WHO.* Extraction of isoflavone malonylglucosides from *Trifolium pretense L.* J Agric Food Chem 2005; 53: 4660–4666
14 *Penalvo JL, Nurmi T, Adlercreutz H.* A simplified HPLC method for total isoflavones in soy products. Food Chem 2004; 87: 297–305
15 *Ralston L, Subramanian S, Matsuno M, Yu O.* Partial reconstruction of flavonoid and isoflavonoid biosynthesis in yeast using soybean type I and type II chalcone isomerases. Plant Physiol 2005; 137: 1375–1388
16 *Elbridge AC, Kwolek WF.* Soybean isoflavones: effect of environment and variety on composition. J Agric Food Chem 1983; 31: 394–396
17 *Liggins J, Bluck LJC, Runswick S, Atkinson C, Coward WA, Bingham SA.* Daidzein and genistein content of fruits and nuts. J Nutr Biochem 2000; 11: 326–331
18 *Liggins J, Bluck LJC, Runswick S, Atkinson C, Coward WA, Bingham SA.* Daidzein and genistein content of vegetables. Br J Nutr 2000; 84: 717–725
19 *Liggins J, Mulligan A, Runswick S, Bingham SA.* Daidzein and genistein content of cereals. Eur J Clin Nutr 2002; 56: 961–966
20 *Tsao R, Yang R, Young JC.* Antioxidant isoflavones in osage orange, *Maclura pomifera* (Raf.) Schneid. J Agric Food Chem 2003; 51: 6445–6451
21 *Wu Q, Wang M, Simon JE.* Analytical methods to determine phytoestrogenic compounds. J Chromatogr B 2004; 812: 325–355
22 *Adlercreutz H, Heinonen SM, Penalvo-Garcia J.* Phytoestrogens, cancer and coronary heart disease. Biofactors 2004; 22: 229–236
23 *Caragay AB.* Cancer preventive foods and ingredients. Food Technol 1992; 46: 65–68
24 *Messina M.* Soy phytoestrogens (isoflavones) and breast cancer. Am J Clin Nutr 1999; 70: 574–575
25 *Anderson JJB, Garner SC.* Phytoestrogens and human function. Nutr Today 1997; 32: 232–239
26 *Park D, Huang T, Frishman WH.* Phytoestrogens as cardioprotective agents. Cardiol Rev 2005; 13: 13–17
27 *Valachovicova T, Slivova V, Sliva D.* Cellular and physiological effects of soy isoflavonoids. Mini Rev Med Chem 2004; 4: 881–887
28 *Branca F, Lorenzetti S.* Health effects of phytoestrogens. Forum Nutr 2005; 57: 100–111
29 *Lin F, Giusti MM.* Effects of solvent polarity and acidity on the extraction efficiency of isoflavones from soybeans (*Glycine max*). J Agric Food Chem 2005; 53: 3795–3800
30 *He J, Zhao Z, Shi Z, Zhao M, Li Y, Chang W.* Analysis of isoflavone daidzein in Puerariae Radix with micelle-mediated extraction and preconcentration. J Agric Food Chem 2005; 53: 518–523
31 *Lee MH, Lin CC.* Comparison of techniques for extraction of isoflavones from the roots of Radix Puerariae: ultrasonic and pressurized solvent extractions. Food Chem 2007; 105: 223–228
32 *Luthria D, Vinjamoori D, Noel K.* Effect of moisture content, grinding, and extraction technologies on assay of crude fat. In: Luthria DL, editor. Oil extraction and analysis: critical issues and comparative studies. Illinois: AOCS Press; 2004: 25–39
33 *Mukhopadhyay S, Luthria DL, Robbins RJ.* Optimization of extraction process for phenolic acids from black cohosh (*Cumicifuga racemosa*) by pressurized liquid extraction. J Sci Food Agric 2006; 86: 156–162
34 *Luthria DL, Biswas R, Natarajan SS.* Comparison of extraction solvents and techniques used for the assay of isoflavones from soybeans. Food Chem 2007; 105: 325–333
35 *Rostagno MA, Palma M, Barroso CG.* Ultrasound-assisted extraction of isoflavones from soy beverages blended with fruit juices. Anal Chim Acta 2007; 597: 265–272
36 *Rostagno MA, Palma M, Barroso CG.* Microwave assisted extraction of soy isoflavones. Anal Chim Acta 2007; 588: 274–282
37 *Rostagno MA, Palma M, Barroso CG.* Solid-phase extraction of isoflavones. J Chromatogr A 2005; 1076: 110–117
38 *Du Q, Li Z, Ito Y.* Preparative separation of isoflavone components in soybeans using high-speed counter-current chromatography. J Chromatogr A 2001; 923: 271–274
39 *Cao X, Tian Y, Zhang T, Li X, Ito Y.* Separation and purification of isoflavones from *Pueraria lobata* by high speed counter-current chromatography. J Chromatogr A 1999; 855: 709–713
40 *Ye H, Chen L, Li Y, Peng A, Fu A, Song H, Tang M, Luo H, Luo Y, Xu Y, Shi J, Wei Y.* Preparative isolation and purification of three rotenoids and one isoflavones from the seeds of *Millettia pachycarpa* Benth by high-speed counter-current chromatography. J Chromatogr A 2008; 1178: 101–107
41 *Xu HN, He CH.* Extraction of isoflavones from stem of *Pueraria lobata* (Willd.) Ohwi using *n*-butanol/water two-phase solvent system and separation of daidzein. Sep Purif Technol 2007; 56: 85–89
42 *Kao TH, Chien JT, Chen BH.* Extraction yield of isoflavones from soybean cake as affected by solvent and supercritical carbon dioxide. Food Chem 2008; 107: 1728–1736
43 *Zuo YB, Zeng AW, Yuan XG, Yu KT.* Extraction of soybean isoflavones from soybean meal with aqueous methanol modified supercritical carbon dioxide. J Food Eng 2008; 89: 384–389
44 *Ksycinska H, Sobik B, Popiolkiewicz J, Polkowski K, Krzeczynski P, Ramza J, Pucko W, Grynkiewicz G.* Determination of new derivative of genistein in culture media by liquid chromatography. J Chromatogr B 2004; 799: 217–231
45 *Grace PB, Mistry NS, Carter MH, Leathem AJ, Teale P.* High throughput quantification of phytoestrogens in human urine and serum using liquid chromatography/tandem mass spectrometry (LC-MS/MS). J Chromatogr B 2007; 853: 138–146
46 *Otieno DO, Rose H, Shah NP.* Profiling and quantification of isoflavones in soy milk from soy protein isolate using extracted ion chromatography and positive ion fragmentation techniques. Food Chem 2007; 105: 1642–1651
47 *Maul R, Schebb NH, Kulling SE.* Application of LC and GC hyphenated with mass spectrometry as tool for characterization of unknown derivatives of isoflavonoids. Anal Bioanal Chem advance online publication 2008; DOI: 10.1007/s00216-008–1884-2008; 4: 1–12

48 *Wilkinson AP, Wähälä K, Williamson G.* Identification and quantification of polyphenol phytoestrogens in foods and human biological fluids. J Chromatogr B 2002; 759: 33–41
49 *Grynkiewicz G, Ksycinska H, Ramza J, Zagrodzka J.* Chromatographic quantification of isoflavones. Acta Chromatogr 2005; 15: 31–65
50 *Vacek J, Klejdus B, Lojkova L, Kuban V.* Current trends in isolation, separation, determenination and identification of isoflavones: a review. J Sep Sci 2008; 31: 2054–2067
51 *Zhai XJ, Qu HB, Shao Q, Cheng YY.* Simultaneous quantification of puerarin and daidzein in rat plasma by high-performance liquid chromatography with post-column modification and fluorescence detection. Chromatgraphia 2007; 66: 43–47
52 *Careri M, Corradini C, Elviri L, Mangia A.* Optimization of a rapid microwave assisted ectraction method for liquid chromatography-electrospray-tandem mass spectrometry determination of isoflavonoid aglycones in soybeans. J Chromatogr A 2007; 1152: 274–279
53 *Lv YQ, Tan TW, Wang MY, Janson JC.* One step rapid determination and purification of puerarin from Radix Puerariae using *n*-octylamine modified poly (methacrylate-co-dimethacrylate) monolith. J Chromatogr B 2008; 871: 1–6
54 *Murphy PA, Barua K, Hauck CC.* Solvent extraction selection in the determination of isoflavones in soy foods. J Chromatogr B 2002; 777: 129–138
55 *Rostagno MA, Araujo JMA, Sandi D.* Supercritical fluid extraction of isoflavones from soybean flour. Food Chem 2002; 78: 111–117
56 *Lee JH, Renita M, Fioritto RJ, Martin SK, Schwartz SJ, Vodovotz Y.* Isoflavone characterization and antioxidant activity of Ohio soybeans. J Agric Food Chem 2004; 52: 2647–2651
57 *Li-Hsun C, Ya-Chuan C, Chieh-Ming C.* Extracting and purifying isoflavones from defatted soybean flakes using superheated water at elevated pressures. Food Chem 2004; 84: 279–285
58 *Klejdus B, Mikelova R, Adam V, Zehnalek J, Vacek J, Kizek R, Kuban V.* Liquid chromatographic-mass spectrophotometric determination of genistin and daidzin in soybean food samples after accelerated solvent extraction with modified content of extraction cell. Anal Chim Acta 2004; 517: 1–11
59 *Klejdus B, Mikelova R, Petrlova J, Potesil D, Adam V, Stiborova M, Hodek P, Vacek J, Kizek R, Kuban V.* Evaluation of isoflavone aglycon and glycoside distribution in soy plants and soybeans by fast column high-performance liquid chromatography coupled with a diode-array detector. J Agric Food Chem 2005; 53: 5848–5852
60 *Achouri A, Boye JI, Belanger D.* Soybean isoflavones: efficacy of extraction conditions and effect of food type on extractability. Food Res Int 2005; 38: 1199–1204