Comprehensive REVIEWS in Food Science and Food Safety

Analysis of Isoflavones in Foods

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Abstract: In recent years the nutritional and bioactive properties of foods are being intensively investigated with a view to control, in addition to food quality, their possible influence on human health. Because of this, there is a growing demand for rapid, selective, sensitive, and validated methods for analysis and quantification. Bioactive plant compounds include those with weak estrogenic activity (phytoestrogens), among which are the isoflavones. Some of the beneficial activities that have been attributed to isoflavones are anticarcinogenic activity, the prevention of cardiovascular disease, the improvement of bone health, and antioxidant activity. The objective of this work is to provide an updated review of the methods used in sample preparation and subsequent analysis for the determination of isoflavones in food samples, including both soybean and soy products, as well as other foods with low isoflavone contents. The review focuses on the most common sample preparation techniques used during the last 10 years, including both conventional solvent extraction and other more recent extraction techniques. Separation and detection methods, including current trends in liquid chromatography analysis, such as the use of monolithic columns or ultra-high-pressure liquid chromatography, are also discussed.

Keywords: capillary electrophoresis, foods, isoflavones, liquid chromatography, sample preparation

Introduction

The nutritional properties of foods are under constant surveillance with a view to control their quality. In recent years there has been increasing interest in knowing, in addition to nutritional properties, the bioactive properties of foodstuffs. This knowledge is relevant not only from the point of view of the consumer, with regards to maintaining health and preventing diseases by using natural foods as part of a healthy diet, but also from an industrial point of view, for the added value of the products (Rostagno and others 2009). Consequently, there is a steadily growing demand for rapid, selective, sensitive, and validated methods for their analysis and quantification.

Bioactive compounds are extra-nutritional constituents that typically occur in small quantities in foods (Kris-Etherton and others 2002), and they are being intensely studied to evaluate potential health benefits. Phytoestrogens (phytochemicals with similar effects as estradiol hormones) are among the bioactive plant compounds with weak estrogenic activity. These include isoflavones, lignans, coumestans, stilbenes, and flavonoids (Wang and others 2002; Wilkinson and others 2002; Wu and others 2004; Akhtar and Abdel-Aal 2006). The phytoestrogens genistein and daidzein from soy bean, biochanin-A from chickpeas, formononetin from clover, and coumestans and lignans derived from flaxseed are the most relevant phytoestrogens to human health. Nearly all of these compounds occur naturally glycosylated in plants, where the bioavailability of these glycoconjugates is different to the corresponding unsubstituted aglycones. Moreover, phytoestrogens can induce estrogenic and antiestrogenic effects in mammals by weakly binding to the nuclear estrogen receptors (ER) α and β; however, they generally have a higher relative binding affinity to ERβ (Sirotkin and Harrath 2014). These compounds have been associated with a lower incidence of steroid-hormone-dependent cancers, including breast, prostate, and colon cancer. Also, phytoestrogens are thought to prevent and treat several dysfunctions and diseases related to aging, such as osteoporosis, and cardiovascular, neurodegenerative, immune and metabolic diseases, as well as symptoms of menopause.

During the past 2 decades, isoflavones have gained significant attention from the scientific community, primarily due to their beneficial effects in the treatment of the symptoms of aging in both females and males.

The basic structure of isoflavone aglycone consists of a 3-phenylchroman skeleton that is hydroxylated in the 4'- and 7-positions. Three types of aglycones mainly exist, depending on the substituents in carbons 5 and 6, called daidzein (De), genistein (Ge), and glycitein (Gle). These 3 isoflavones can exist as 7-O-glucosides (daidzin (Di), genistin (Gi), and glycitin (Gly)), which can be esterified at the 6''-O-position of the glucose ring with acetyl or malonyl groups (Peñalvo and others 2004; Luthria and others 2007), generating the corresponding acetylglucosides (ADi, AGi, AGly) and malonylglucosides (MDi, MGi, MGly). The 4' hydroxyl group of the chroman skeleton can be methylated, resulting in the aglycones formononetin (For) and biochanin-A (Bio), precursors of daidzein and genistein, respectively (Mazur 1998).

Isoflavones are widely distributed within the plant kingdom, but they accumulate predominantly in plants of the Leguminosae

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doi: 10.1111/1541-4337.12325

family. The main sources of isoflavones are soy and soy-derived products. Other natural sources of isoflavones are red clover, kudzu, alfalfa, chickpeas, beans, and other which also belong to the Fabaceae family. Moreover, small amounts of isoflavones are also contained in other plant products such as fruits, vegetables, nuts, and cereals (Liggins and others 2000a, 2000b, 2002). In plants, isoflavones are usually found in conjugated forms, such as glucosides (acetylglucosides or malonylglucosides), which are not absorbed intact across the enterocyte of healthy adults. Their bioavailability requires hydrolysis to the corresponding aglycones, by intestinal β-glucosidases, which are then absorbed in the intestinal wall due to their high liposolubility and lower molecular weight (Setchell and others 2002).

Due to the great interest in isoflavones during the past decades, there has been a dramatic increase in the development of analytical methods for the determination of isoflavones in plant samples, foods, and other biological matrices, as demonstrated by the number of reviews published up to 2009 (Grynkiewicz and others 2005; Dentith and Lockwood 2008; Vacek and others 2008; Luthria and Natarajan 2009; Mortensen and others 2009; Rostagno and others 2009; Valls and others 2009). More recently, a review on the analytical methods used to quantify isoflavones in cow milk (Daems and others 2016); review articles regarding the analysis of polyphenols (Motilva and others 2013; Lucci and others 2017), flavonoids (de Villiers and others 2016), and isoflavonoids (Raju and others 2015), using liquid chromatography coupled to mass spectrometry, have also been published.

Two Official Methods of Analysis have been developed by AOAC International for the determination of isoflavones in soy beans, dietary supplements, and soy foods: the AOAC Official Method 2001.10 (AOAC 2001), based on extraction, alkaline hydrolysis, and liquid chromatography with UV detection (LC-UV), for the determination of isoflavone glycosides and aglycones; and the AOAC Official Method 2008.03 (AOAC 2008), based on extraction and LC-UV, for the determination of isoflavone glycosides, malonyl-, and acetyl-glycosides, and aglycones. Recently, Phillips and others (2017) developed 2 analytical methods, based on liquid chromatography with absorbance detection and with isotope-dilution mass spectrometry, for the determination of 6 isoflavones (Di, Gi, Gly, De, Ge, Gle) in soy standard reference materials (SRMs). Both methods were based on basic hydrolysis to cleave malonyl- and acetyl-glycosides and an internal standard approach for quantification. These authors analyzed 4 SRMs produced by the U.S. National Institute of Standards and Technology. The results obtained by using both methods coincided, and they were used to assign isoflavone content values to the matrix-based SRMs.

The objective of this article was to provide an overview of the literature published over the last 10 y on the determination of isoflavones in foods, mainly using liquid chromatography, but also including articles referring to other used separation techniques. We also provide a review of the sample preparation procedures currently employed to carry out such analysis.

Analysis of Isoflavones in Foods

Generally, in the analysis of isoflavones in food 3 key stages can be distinguished: sample preparation, separation of individual analytes, and detection and quantification. The most commonly used techniques for carrying out each of these steps in the determination of isoflavones are described below.

Sample Preparation

Sample preparation is a key step in the optimization of any analytical method owing to its effect on the reliability and accuracy of the determinations. The most common sample treatments include drying, homogenization, sieving, extraction of the target compounds, elimination of the interferences, preconcentration, hydrolysis, and derivatization. The nature of the sample and analyte being studied influences the type of procedure, technique, or conditions that are to be used.

Most extraction methods are based on conventional techniques such as liquid–liquid, solid–liquid, or the Soxhlet extraction (Table 1 and 2). However, other techniques have been reported for the isolation of isoflavones from food samples, such as solid-phase extraction (SPE), ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), and supercritical fluid extraction (SFE) (Table 3). The development of these techniques is closely linked to the search for faster, more extractive, specific, and selective methods capable of extracting smaller samples. On the other hand, new trends in analytical chemistry are aimed at miniaturizing the sample preparation and minimizing the use of organic solvents, as well as the automation of the process.

Direct solvent extraction

In solvent extraction, polarity and volume of the solvent, temperature, duration of extraction, and sample type and amount are the main factors that influence the efficiency of the process.

Solid samples need to be ground, whereas liquid samples do not. However, in some cases liquid samples are freeze-dried and treated the same as solid samples (Otieno and others 2007; Jung and others 2008; Toro-Funes and others 2012, 2014a, 2014b; Fahmi and others 2014).

The most commonly used solvents for the extraction of food isoflavones are methanol (MeOH), ethanol (EtOH), acetonitrile (MeCN), and acetone (ACE), either pure or mixed with water in different proportions, and with or without the addition of small amounts of acids. The choice of extraction solvent depends on both sample type and the analytes to be determined.

Soybeans and soybean products. In the case of soybeans and soy products, the most abundant form of isoflavones present in the sample depends on the type of treatment to which the sample has been subjected.

Thus, in soybeans and nonfermented soy products (soy protein, soymilk derivatives, and so on) the predominant isoflavones are β-glucosides and malonyl-β-glucosides (the latter representing 70% to 80% of the total amount of isoflavones), while fermented soy products (such as miso, tempeh) contain mainly aglycones (Peñalvo and others 2004; Lee and Lee 2009; Hong and others 2011). With respect to this, Park and others (2012) studied the modification of the isoflavone distribution in a fermented soy food using almond powder with high β-glucosidase activity. These authors observed that the malonyl derivatives of isoflavones decreased and aglycones increased when almond powder was added to the sample within 48 h.

Lee and others (2015) compared the extraction of isoflavones from fresh and fermented soybeans, soymilk, and tofu using 80% MeOH and 80% MeCN with and without HCl. In this study, they showed that 80% MeOH was significantly more effective for the extraction of malonyl and acetyl glucosides, whereas 80% MeCN was used for the extraction of isoflavone glucosides and aglycones. The addition of hydrochloric acid to both extraction

Table 1–Analytical methods for extraction of isoflavones from soybean and soybean in products using direct solvent extraction.

Sample	Solvent mixture	Agitation system, time and temperature	Separation and quantification	References
Soybean products (soybean, tofu, fermented soy-beans, and soymilks)	Comparison between 80% MeOH, 80% MeOH in 0.1 M HCl, 80% MeCN, and 80% MeCN in 0.1 M HCl	Shaking at 500 rpm for 2 h	HPLC–ESI-TOF-MS	Lee and others (2015)
Freeze-dried soymilk	80% (v/v) aqueous MeOH	Shaking and heating in a water bath at 60 °C for 2 h.	LC-UV	Fahmi and others (2014)
Soybean (*Glycine max*), wild soybean (*Glycine soja*) and bean products	80% (v/v) aqueous MeOH	Ultrasonic extraction at room temperature for 1 h	LC-ESI-MS (IT) LC-DAD	Hong and others (2011)
Soy-based nutritional supplements	80% (v/v) aqueous MeOH with 150 mg of zinc sulfate heptahydrate to prevent possible hazy extracts	Continuous shaking at 600 rpm and room temperature for 1h using a laboratory orbital shaker	HPLC-tunable ultraviolet (TUV) detection	Fiechter and others (2010)
Soybean flour	80% (v/v) aqueous MeOH	Shaking for 1 h at room temperature	HPLC-DAD for determination; HPLC-MS(/MS) (Q-TOF) for confirmation	Aguiar and others (2012)
Extracts from different stages of soybean processing	80% (v/v) aqueous MeOH	Sonication for 10 min	HPLC–DAD	Gasparetto and others (2012)
Soy-based infant formulas	80% (v/v) aqueous MeOH	Extraction with an Ultra-Turrax extractor at 22000 rpm for 1 min (3 times); ultrasonication for 15 min; concentration by rota-evaporation; SPE with Strata-X cartridges	LC–ESI-MS	Fonseca and others (2014)
Soy-based nutritional supplements	80% (v/v) aqueous MeOH	Shaking for 1 min in a vortex; stirring in a rotatory agitator for 2 h (twice)	UHPLC–Orbitrap–MS	López-Gutiérrez and others (2014)
Soy isoflavone concentrate	80% (v/v) aqueous MeOH	Sonication at 40 °C for 10 min	HPLC–DAD–ESI-MS	Verardo and others (2015)
Soybean	80% (v/v) aqueous MeOH	Homogenization using a Polytron homogenizer for 15 s; purification with Oasis® HLB SPE cartridges	HPLC-ESI-MS/MS	Cavaliere and others (2007)
Soymilk made from soy protein isolate	MeOH	Reflux on a heating mantle for 1 h	HPLC-ESI-MS/MS	Otieno and others (2007)
Soybean and fermented soy food with almond powder	0.1 M HCl:MeCN:H_2O (2:7:3, v/v/v)	Vortex mixing or shaking for 2 h	HPLC-UV	Lee and Lee (2009); Park and others (2012)
Seed, embryo, cotyledon and seed coat of cooked-with-rice and soybean	0.1 M HCl:MeCN (2:10, v/v)	Sonication for 2 h at room temperature	HPLC-UV	Kim and others (2007, 2014a, 2014b)
Powdered infant formulas	MeCN	Double-extraction in ultrasound bath for 15 min; purification by SPE with OASIS® HLB cartridges	LC–ESI-MS/MS	Maggioni and others (2013)
Foods (soy bean, black bean, red bean and 3 soy-bean pastes)	70% aqueous MeOH:DMSO (8.4:3.6, v/v)	First addition of DMSO and vortex mixing for 5 min; then addition of 70% methanol and vortex mixing again for 5 min.	UHPLC-PDA	Shim and others (2015)
Tofu-type soybeans	70% aqueous MeOH for qualitative analysis; hydrolysis with 95% aqueous EtOH:concentrated HCl (70:8, v/v) for quantitative analysis	Sonication at 0 °C for 20 min for qualitative analysis; reflux for 2.5 h for quantitative analysis	HPLC-PDA-ES-MS	Shen and others (2012)
Nutritional supplements of soy	80% aqueous MeOH:DMSO (24:1, v/v)	Extraction for 20 min in an ultrasonic bath at 60 °C	HPLC-UV FT-IR	Mulsow and others (2015)
Dietary supplements	MeCN:H_2O:DMSO (58.5:39.0:2.5, v/v/v)	Extraction at room temperature for 1 h	HPLC-UV	Collison (2008)

(*Continued*)

Table 1–Continued.

Sample	Solvent mixture	Agitation system, time and temperature	Separation and quantification	References
Soybean	Comparison between 7 different solvent mixtures: MeCN:H_2O (58:42, v/v), DMSO:MeCN:H_2O (5:58:37, v/v/v), EtOH:H_2O (70:30, v/v), MeOH:H_2O (90:10, v/v), DMSO:EtOH:H_2O (5:70:25, v/v/v), H_2O and genapol:H_2O (5:95, v/v)	Comparion between stirring, shaking, vortexing, sonication, Soxhlet and PLE	HPLC-DAD	Luthria and others (2007)
Transgenic soybean BRS 243 RR	70% aqueous EtOH containing 0.1% acetic acid	Vortexing for 30 s for 1 h every 15 min; sonication at room temperature for 30 min	HPLC-UV	Alezandro and others (2008)
Soy flour variety BRS 25	70% aqueous EtOH containing 0.1% acetic acid	Shaking at 25 °C for 1 h, at intervals of 15 min; Sonication for 30 min	HPLC-UV	Giaretta and others (2015)
Soy foods and supplements	70% aqueous EtOH	Stirring or shaking for 30 min at room temperature (twice) for solid samples; mixing for 2 min for soy milk	HPLC-UV	Yanaka and others (2012)
Soybean flour, texturized soy protein, soy fibre, powdered soy milk and liquid soy drink	50% aqueous EtOH for solid samples, MeOH for liquid samples	Sonication for 20 min at 60 °C. Liquid samples were cleaned/concentrated using SPE with Strata X cartridges	HPLC-DAD	Rostagno and others (2007a)
Yellow soybean and texturized soy protein	50% aqueous EtOH	Extraction on a multi-frequency ultrasonic bath for 20 min at 60 °C.	HPLC-DAD	Manchón and others (2010)
Soybean	Comparison between different solvent mixtures of H_2O, ACE, EtOH and MeCN	Extraction in ultrasonic bath at 60 °C for 10 min	UHPLC-DAD	Yoshiara and others (2012)
Soybean	UHQ:ACE:EtOH (1:1:1, v/v/v)	Extraction in ultrasonic bath at 60 °C for 10 min	UHPLC-DAD	Quinhone Júnior and Ida (1989); Borges and others (2016)
UHT soy milk	ACE:0.1 M HCl (5:1 v/v)	Stirring in a magnetic plate for 2 h at room temperature	UHPLC-UV	Toro-Funes and others (2012, 2014a, 2014b)
Soymilk	MeOH	Vortexing for 30 s; sonication for 5 min	UPLC-PDA-ESI-MS	Zhang and others (2017)
Soy beverages blended with fruit juices	EtOH	Sonication at 45 °C for 20 min	HPLC-UV	Rostagno and others (2007b)
Soy drink	EtOH	Extraction using an automatic inversion mixer for 30 min	CZE–ESI-MS	Bustamante-Rangel and others (2012)
Isoflavone-enriched milk and juice	Precipitation solution:MeCN:H2O (2:2:10, v/v/v)	Vortexing for 1 min at room temperature and darkness for 15 min	HPLC-DAD	Zafra-Gómez and others (2010)
Soy food preparations	90% aqueous MeOH	Soxhlet extraction using a 2-step temperature program (first/second step: temperature of cooling/heating block 130/120 °C for 30 min, cooling/heating block to 30/30 °C for 5 min)	HPLC-ESI-MS	Klejdus and others (2007)
Soybean meal and fermented soybean meal	80% aqueous EtOH	Soxhlet extraction under reflux	MECC	Xiao and others (2011)
Glycine max beans	80% aqueous EtOH	Soxhlet extraction at 70 to 80 °C for 4 h; addition of HCL and heating at 80 °C for 2 h	HPLC-DAD	Yatsu and others (2014)
Glycine max dry extract	MeCN:H_2O (10:6, v/v)	Shaking for 2 h at 60 °C for direct analysis; or followed by hydrolysis with HCl for 2 h at 80 °C	HPLC-DAD	

Table 2–Analytical methods for extraction of isoflavones from other *Fabaceae* and vegetables using direct solvent extraction.

Sample	Solvent mixture	Agitation system, time and temperature	Separation and quantification	References
Grains and leaves of several leguminous plants	80% (v/v) aqueous MeOH	Extraction at 26 °C for 1 h	HPLC–DAD and HPLC–ESI-MS	Aguiar and others (2007)
Legumes (lentils, chickpeas, beans)	80% (v/v) aqueous MeOH	Stirring 3 times using an Ultra-Turrax homogenizer for 10 s. Purification by SPE using Strata C18-E cartridges	UHPLC–ESI-MS/MS	Vila-Donat and others (2015)
Legumes (chickpeas, beans, lentils)	MeOH:0.1 M HCl (10:2, v/v)	Sonication for 2 h at room temperature	LC–ESI-MS/MS	Konar and others (2012a)
Chickpea protein concentrates	HCl:MeOH:H_2O (1:80:20, v/v/v), ethyl ether and ethyl acetate	Shaking for 30 min at room temperature with HCl:MeOH:H_2O, extraction with ethyl ether and ethyl acetate (3 times each)	HPLC-UV	Megías and others (2016)
Chickpeas	50% aqueous MeOH and 70% aqueous acetone	Magnetic stirring at room temperature for 60 min with methanol:water (50:50, v/v), sonication, centrifugation and reextraction with acetone:water (70:30, v/v)	HPLC–DAD– QTOF-MS	Mekky and others (2015)
Groundnut	80% (v/v) aqueous EtOH	Shaking for 1 h	HPLC-DAD	Nara and others (2011)
Chickpeas	70% (v/v) aqueous EtOH	Extraction twice for 3 h at 70 °C	HPLC–DAD– ESI-MS	Gao and others (2015)
Chickpea yogurt	70% (v/v) aqueous EtOH	Blending for 4 h at 70 °C in water bath	HPLC-UV	Fu and Zhang (2013)
Red clover	MeOH:H_2O:HCl (70:10:10, v/v/v)	Refluxing at 90 °C for 1 h	HPLC-DAD	Gikas and others (2008)
Trifolium species	80% (v/v) aqueous MeOH acidified to pH 3 with trifluoroacetic acid	Refluxing at 85 °C for 15 min	HPLC-DAD	Renda and others (2013)
Trifolium scabrum L.	80% (v/v) aqueous MeOH	Extraction at room temperature; preparative LC	NMR and UPLC-DAD-ESI-MS	Kowalska and others (2013)
Red clover	MeOH:H_2O:1 M HCl (17:3:2, v/v/v)	Refluxing 3 times for 60 min	MECC	Zhang and others (2007)
Radix Puerariae	MeOH	Sonication for 30 min	HPLC-DAD-ESI-IT-TOF-MS	Niu and others (2012)
Radix Puerariae	MeOH	Sonication for 30 min	MEKC / CZE	Xiao and others (2015a, 2015b)
Medicago species	Three different solvents: EtOH, EtOH:H_2O (1:1, v/v) and H_2O	Maceration 30 min at 40 °C	HPLC-DAD	Rodrigues and others (2014)
Mung bean	50% (v/v) aqueous EtOH	Homogenization using an oscillation ball mill; sonication for 40 min	UPLC–ESI–MS/MS	Prokudina and others (2012)
Azorella madreporica Clos.	MeOH	Extraction (3 times) at room temperature for 2 days each	HPLC-PDA-ESI-TOF-MS	Bórquez and others (2013)
Chia seeds	70% (v/v) aqueous MeOH containing 0.1% acetic acid	Incubation for 1 h at 30 °C under mechanical shaking	HPLC-DAD	Martínez-Cruz and others (2014)
Sour cherry	MeOH:H_2O:formic acid (60:39:1, v/v/v)	Sonication for 40 min	HPLC-DAD-ESI-qToF-MS	Abrankó and others (2015a, 2015b)
Vegetables and fruits	80% (v/v) aqueous MeOH	Agitation for 30 min with a rotary shaker	UHPLC–MS/MS	Alarcón-Flores and others (2013, 2014)
Coffee	MeOH	Vortexing for 180 s; centrifugation and freezing overnight at –24 °C; purification on SPE Strata C18 cartridges	HPLC-MS/MS	Caprioli and others (2016)
Plant materials (*Trifolium pratense, Iresine herbstii, Ononis spinosa)*	90% (v/v) aqueous MeOH	Soxhlet extraction using a 2-step temperature program (first/second step: temperature of cooling/heating block 130/120 °C for 30 min, cooling/heating block to 30/30 °C for 5 min)	HPLC-ESI-MS	Klejdus and others (2007)
Legumes used as pulses, pasture legumes and other plants belonging to the Leguminosae family	90% (v/v) aqueous MeOH	Soxhlet extraction for 60 min (10 min immersion and 50 min washing)	HPLC-DAD	Leuner and others (2013)

Table 3–Analytical methods for extraction of isoflavones using other extraction techniques.

Sample	Extraction technique	Extraction conditions	Separation and quantification	References
Soybean	MAE	50% (v/v) aqueous EtOH, 50 °C, 20 min	HPLC–UV	Rostagno and others (2007c)
Soybean	MAE	90% (v/v) aqueous EtOH, 50 °C, 20 min	HPLC–UV	Jiao and others (2012)
Soybean	MAE	80% (v/v) aqueous MeCN, sonication for 15 min; addition of 12 M HCl (1:2, v/v), MAE 1 min	HPLC–ESI-MS/MS	Careri and others (2007)
Soybean flour	Continuous MAE	EtOH, 73 °C, 8 min	HPLC-UV	Terigar and others (2010)
Radix Astragali	MAE	MeOH, 300 W, 4 min	HPLC-UV-ELSD	Song and others (2007)
Peanuts	MAE	80% (v/v) aqueous EtOH, sonication for 2 h; MAE at 70 °C intermittently for a total of 30 s with power off for 10 s in between 6 s of irradiation	HPLC-UV	Chukwumah and others (2007)
Soybean	Comparison between different extraction techniques	PLE conditions: DMSO:EtOH:H_2O (5:70:25, v/v/v), 100 °C, 6.9 MPa, 7 min static time, 3 extraction cycles	HPLC-DAD	Luthria and others (2007)
Trifolium species	Comparison between UAE, DSE and PLE	PLE conditions: 75% (v/v) aqueous MeOH, 125 °C, 10 MPa, 5 min static time, 3 extraction cycles	HPLC-UV	Zgórka (2009)
Radix Puerariae	Comparison between UAE, DSE and PLE	PLE conditions: 95% (v/v) aqueous EtOH, 100 °C, 9.6 MPa,10 min static time, 1 extraction cycle	HPLC-UV	Lee and Lin (2007)
Soybean flakes	PLE	80% (v/v) aqueous EtOH, 110 °C, 551 kPa, 25 mL/min flow rate	HPLC-UV	Chang and Chang (2007)
Chickpeas and lentils	PLE	50% (v/v) (for chickpeas) or 75% (v/v) (for lentils) aqueous MeOH, 11 MPa, 5 min static time, 3 extraction cycles	HPLC-DAD	Delgado-Zamarreño and others (2012a)
Soybean flour and soy protein isolate	PWE	Water, 10 MPa, 114 °C for 2 min (for soybean flour) or 120 °C for 14 min (for soy protein isolate)	HPLC-UV	Moras and others (2017)
Plants (*Matricaria recutita, Rosmarinus officinalis, Foeniculum vulgare*, and *Agrimonia eupatoria* L.)	Comparison between SFE, PLE, MSPD, UAE, Soxhlet and SPE	PLE conditions: MeCN, 100 °C, 10 MPa, 15 min extraction time, 2 extraction cycles SFE conditions: CO_2 with 5% MeOH as modifier, 70 °C, 35 MPa, 30 min extraction time	HPLC-UV	Bajer and others (2007)
Soybean hypocotyl	SFE	CO_2 with 10% mol 80% (v/v) MeCN as modifier, 60 °C, 38 MPa, 15 min extraction time	HPLC-UV	Araújo and others (2007)
Soybean cake	SFE	CO_2 with EtOH:H_2O (70:30,v/v) as modifier, 60 to 80 °C, 35 MPa, triplicate extraction: 10 min static extraction followed by 20 min dynamic extraction	HPLC-DAD	Kao and others (2008)
Soybean meal	SFE	CO_2 with 7.8% mass 80% (v/v) aqueous MeOH as modifier, 40 °C and 50 MPa, 9.80 kg/h flow rate	HPLC-UV	Zuo and others (2008)
Sea algae	SFE	CO_2 with 3% (v/v) MeOH/H_2O (9:1, v/v) as modifier, 40 °C, 35 MPa, 60 min	Fast chromatography-MS/MS	Klejdus and others (2010)

(*Continued*)

Table 3–Continued.

Sample	Extraction technique	Extraction conditions	Separation and quantification	References
Legumes	QuEChERS	Two-step extraction: 70% (v/v) aqueous MeCN for 5 min, MeCN for 5 min; $MgSO_4$:NaCl (4:1, w/w), without d-SPE. Agitation systems: vortex, ultrasound probe and thermostatted tray shaking	UHPLC-ESI-MS/MS	Delgado-Zamarreño and others (2012b); Bustamante-Rangel and others (2014)
Soy biscuits	QuEChERS	50% (v/v) aqueous MeCN, $MgSO_4$:NaCl (4:1, w/w) and citrate buffer as salts, mixing for 5 min, d-SPE with $MgSO_4$:silica sorbent:PSA:C18 (150:150:25:25, w/w/w/w)	CE-ESI-MS	Bustamante-Rangel and others (2013)
Soybeans	QuEChERS	70% (v/v) aqueous MeCN, $MgSO_4$:NaCl (4:1, w/w), vortexing for 1 min, sonication for 20 min, without d-SPE	LC-Q-TOF-MS	Ding and others (2016)
Soybean	MSPD	Silica gel as sorbent, MeOH at pH 3 as elution solvent	HPLC-UV	Barfi and others (2009)
Red clover leaves	MSPD	C18 as sorbent, dichloromethane:methanol (25:25, v/v) as elution solvent	HPLC-DAD	Visnevschi-Necrasov and others (2009)
Medicago spp. and other *Leguminosae* plants	MSPD	C18 as sorbent, 90% (v/v) aqueous MeOH as elution solvent	HPLC-DAD	Visnevschi-Necrasov and others (2015); Barreira and others (2015); Cunha and others (2012)
Soy drinks	SPME	Polydimethylsiloxane/divinylbenzene fiber, exposure time 20 min, desorption in static mode by soaking in the mobile phase for 15 min	SPME-HPLC-DAD	Aresta and others (2016)
Soymilk	MSPE	Baicalin functionalized core-shell magnetic nanoparticles, vortexing 5 min, elution with MeOH at 60 °C	HPLC-ESI-MS/MS	Qing and others (2013)
Soymilk	MSPE	Chitosan microparticles, stirring at 30 °C for 10 min, elution with 40 % MeOH	HPLC-UV	Guo and others (2017)

solvents (MeOH and MeCN) significantly reduced the extraction efficiency.

The most frequently used solvent mixture for the extraction of the 12 major forms of isoflavones from soybeans and their derivatives is 80% aqueous methanol (Cavaliere and others 2007; Fiechter and others 2010; Hong and others 2011; Aguiar and others 2012; Gasparetto and others 2012; Fahmi and others 2014; Fonseca and others 2014; López-Gutiérrez and others 2014; Verardo and others 2015). In very few cases pure methanol is used to carry out the extraction (Otieno and others 2007). However, acetonitrile combined with hydrochloric acid is still used as the solvent in many cases for the extraction of isoflavones from soybeans and soy products (Kim and others 2007, 2014a, 2014b; Lee and Lee 2009; Park and others 2012). Pure MeCN has been used by Maggioni and others (2013) for the analysis of genistein, genistin, and fungicides in infant formulas. The samples were double-extracted with 10 and then 5 mL of acetonitrile for 15 min each in an ultrasound bath, before their purification using solid-phase extraction.

Shim and others (2015) evaluated the use of dimethylsulfoxide (DMSO) as a pre-extraction solvent, due to the ability of DMSO to be strongly associated with organic acids, alcohols, and phenols via hydrogen-bonding between the lone pairs on the DMSO oxygen and the "bound" hydrogen of the substrate's hydroxyl group. These authors added DMSO to the homogenized sample (soy bean, black bean, red bean, and soybean pastes) and vortexed the mixture for 5 min before adding 70% methanol. They showed that the addition of DMSO allowed the sonication step to be excluded, without the loss of the target compounds, and it reduced pretreatment time when compared with the method proposed by Shen and others (2012). The latter included the addition of 70% methanol followed by the use of sonication at 0 °C for 20 min. A procedure similar to that proposed by Shim and others (2015) was used by Mulsow and others (2015) for the extraction of isoflavonol glycosides in soy nutritional supplements. Aqueous acetonitrile containing a small amount of dimethylsulfoxide (Collison 2008) has also been used in an interlaboratory study for the determination of total soy isoflavones in dietary supplements, dietary supplement ingredients, and soy foods.

Luthria and others (2007) compared 4 different solvent mixtures commonly employed for the extraction of isoflavones from soybean (58% MeCN, 70% EtOH, 90% MeOH, and water), and evaluated the influence of the addition of 5% dimethylsulfoxide (DMSO) to 58% MeCN and 70 % EtOH, and 5% genapol to water. They observed that optimum extraction of the total isoflavones content was obtained with a DMSO:EtOH:water (5:70:25, v/v/v) solvent mixture. The authors also found significant differences among the extraction of individual isoflavones with respect to the solvent mixtures assayed: the highest yields of glucosides (Di, Gi, and Gly) were obtained with 90% MeOH, whereas the maximum yields for the rest of forms (ADi, AGi, AGly, MDi, MGi, MGly, De, Ge, and Gle) were obtained with DMSO:EtOH:water (5:70:25). The detection of all 12 isoflavones in measurable quantities was only achieved with 2 extraction solvent mixtures: DMSO:EtOH:water (5:70:25) and DMSO:MeCN:water (5:70:25).

Ethanol:water (70:30, v/v) has been used by Alezandro and others (2008) and Giaretta and others (2015) for the determination of isoflavones from transgenic soybean BRS 243 RR and the soy flour variety BRS 257, respectively. In both cases, the sample was defatted with hexane prior to extraction with ethanol, which also contained 0.1% acetic acid. The addition of acetic acid resulted in more defined chromatographic peaks. In addition, 70% aqueous ethanol without acidification has also been used by Yanaka and others (2012) for the extraction of 15 isoflavones (the above mentioned 12 plus succinyl-glucosides) from soy foods and supplements from different countries. Rostagno and others (2007a) extracted the 12 main isoflavones from soybean flour, texturized soy protein, soy fiber, and powdered soy milk using 50% ethanol in water. The same solvent was used by Manchón and others (2010) to extract isoflavones from yellow soybean and texturized soy protein samples. In both cases, the extractions were performed in an ultrasonic bath at 60 °C for 20 min. On the other hand, the use of EtOH to carry out the extraction has additional advantages, such as being less toxic, environmental friendly, and of low cost.

Another study on solvent optimization in the extraction of soy isoflavones was published by Yoshiara and others (2012). These authors used the simplex-centroid mixture design with 4 solvents of varying polarity (water, acetone, ethanol, and acetonitrile) in 15 different mixtures (pure solvents, six 1:1 binary mixtures, four 1:1:1 ternary mixtures, and one 1:1:1:1 quaternary mixture). The extraction of isoflavones from defatted soy flour was carried out in an ultrasonic bath at 60 °C for 10 min. The results obtained showed that the malonyl-glycosidic forms (high polarity) and the total forms were best extracted with water:acetone:ethanol (2:1:1). Glycosidic isoflavones (intermediate polarity) were best extracted with water:acetone:acetonitrile (2:1:1), and the aglycone forms (low polarity) were optimally extracted with water:acetone (1:1). Mixtures with water:acetone:ethanol (1:1:1, v/v/v) have been used by Quinhone Júnior and Ida (2015) to study the effect of the time of germination on different forms of soybean isoflavones. These authors observed a predominance of malonylglucosides in BRS 284 soybean seeds. Borges and others (2016), using the same extraction conditions, evaluated the effect of the processing conditions of soybean tempeh on the contents of β-glycoside isoflavones and on their bioconversion into aglycones.

Toro-Funes and others (2012, 2014a, 2014b) used acetone:HCl 0.1 M (5:1, v/v) for the extraction of aglycones, glycosides, and acetyl and malonyl glycosides of the main isoflavones in soy milk. Prior to extraction, the samples were frozen at –80 °C for 24 h and then lyophilized.

In view of these results, it can be concluded that the preferred extraction solvent is 80% aqueous methanol, nonacidified, since it provided the best yields in the recovery and did not produce transformations of the naturally occurring forms of isoflavones present in the food.

Other Fabaceae and vegetables. Isoflavones are also present in other plants of the Fabaceae family, such as *Trifolium pratense* (red clover), *Pueraria lobata* (kudzu), *Medicago sativa* (alfalfa), *Cicer arietinum* (chickpeas), *Phaseolus vulgaris* (beans), and others.

In western countries, soybeans and soy products are not traditional foods and are not widely consumed. In contrast, other types of legumes, such as chickpeas, lentils, and beans, are frequently consumed; these pulses mainly contain aglycones (De, Ge, Gle, Bio, For) and glucosides (Di, Gi, Gly). The extraction of these samples, similar to soybean samples, is usually carried out using methanol:water mixtures, generally in a 80:20 (v/v) ratio (Aguiar and others 2007; Vila-Donat and others 2015), in some cases with acidification of the extraction mixture (Konar and others 2012a; Megías and others 2016). Other mixtures such as methanol:water 50:50 (v/v) (Mekky and others 2015), ethanol:water (80:20, v/v) (Nara and others 2011), and (70:30, v/v) (Fu and Zhang 2013; Gao and others 2015) have also been used.

In red clover and other species of Trifolium the main isoflavones are biochanin A and formononetin, with smaller concentrations of

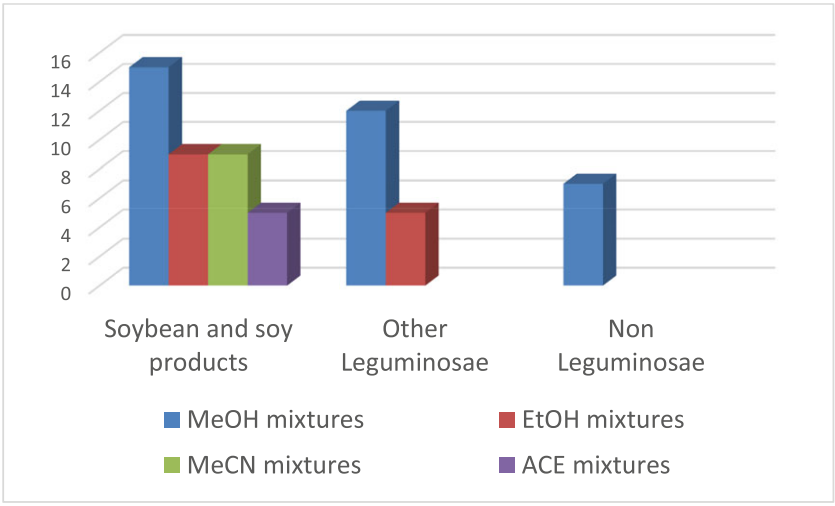

Figure 1–Comparison between the most commonly employed solvent mixtures in the extraction of isoflavones.

daidzein and genistein. Generally, the extraction of the isoflavones from these matrices is achieved using 80% aqueous methanol; however, in some cases an acidic medium is used. So, Gikas and others (2008) quantified these isoflavones in the arterial part of red clover from samples collected at the flowering, vegetative, and fruiting stages, with the aim of determining which of the 3 growth stages contained the highest isoflavone content. The authors mixed the pulverized sample with MeOH:H_2O:HCl (7:1:1, v/v/v) and refluxed the mixture at 90 ºC for 1 h. Renda and others (2013) used an aqueous methanolic mixture acidified with trifluoroacetic acid for the extraction of isoflavones from 13 different Trifolium species. In this case, the optimum extraction temperature was 85 ºC.

Radix Puerariae, the dry root of Pueraria lobata, also contains large amounts of isoflavones, including puerarin, daidzin, genistein, and biochanin A. These samples are generally extracted with pure methanol and subjected to an ultrasonic bath for 30 min (Niu and others 2012; Xiao and others 2015a, 2015b).

Rodrigues and others (2014) evaluated the isoflavone contents of various *Medicago* species and found that the most abundant isoflavones were genistin, daidzein, and genistein. The authors examined 3 different solvents (ethanol, ethanol:water (1:1), and distilled water), and they observed that the extractability of the compounds significantly increased as the solvent became more alcohol-based.

Isoflavones, along with other isoflavonoids and phenylpropanoids, have also been determined in other Fabaceae, such as Mung bean (*Vigna radiata*) sprouts (Prokudina and others 2012), where lyophilized sprouts were mixed with 50% ethanol and sonicated for 40 min.

Isoflavones have been analyzed in plants belonging to families other than Leguminosae, such as *Azorella madreporica* which contain genistein derivatives (Bórquez and others 2013), and chia seeds (*Salvia hispanica L.*), which contain daidzin as the main isoflavone, as well as genistein, genistin, glycitin, and glycitein (Martínez-Cruz and Paredes-López 2014). In these cases, extraction was carried out using methanol and methanol:water (70:30,v/v) containing 0.1% of acetic acid, respectively.

Isoflavones have also been found in fruits and vegetables. For example, Abrankó and others (2015a, 2015b) extracted genistein glycoconjugates from sour cherry with methanol:water:formic acid (60:39:1) and Alarcón-Flores and others (2013, 2014) determined various phytochemicals, including isoflavones, in vegetables such as tomato, broccoli, and carrot, using methanol:water (80:20, v/v) as the solvent.

Caprioli and others (2016) developed an analytical method for determining 5 isoflavones present in espresso and ground coffee (Gi, De, Ge, For, and Bio). These authors tested 4 extraction methods for isoflavones from espresso coffee samples: dilution, purification with SPE cartridges, defatting with hexane followed by SPE, and extraction with methanol, freezing and SPE. The last method provided the best results in terms of reliability and precision.

Liquid samples. As discussed above, liquid samples are often treated as solid samples, following a lyophilization process. In other cases, extraction is carried out directly from the liquid sample with methanol (Rostagno and others 2007a; Zhang and others 2017) or ethanol (Rostagno and others 2007b; Bustamante-Rangel and others 2012; Yanaka and others 2012). For these samples, the most important parameter for the extraction of isoflavones is the sample:solvent ratio, which can vary between 0.2:1 and 4:1, and the extraction time is usually very short (30 s to 30 min).

Occasionally, an initial precipitation step is carried out to remove proteins and lipids, such as in the determination of the isoflavone content of supplemented cow milk and juices, as performed by Zafra-Gómez and others (2010). These authors used a precipitation solution prepared by dissolving zinc acetate, phosphotungstic acid polyhydrated and glacial acetic acid in water. The extraction solvent was acetonitrile:water.

Figure 1 shows a comparison between the most commonly employed solvent mixtures. As can be seen, mixtures containing methanol are the most frequently used in all cases.

Extraction technique. Individual direct solvent extraction methods can differ with regard to the extraction solvent used, the extraction technique, temperature, time, and number of extractions. Stirring, shaking, vortexing, and sonication are the most commonly used extraction techniques, as can be seen in Table 1 and 2.

The Soxhlet method has also been used for the extraction of isoflavones from solid samples of soybeans, soy products, and

legumes. The extraction is performed using 90% aqueous MeOH (Klejdus and others 2007; Leuner and others 2013) or 80% aqueous ethanol (Xiao and others 2011; Yatsu and others 2014) as the solvents. Klejdus and others (2007) proposed a modified Soxhlet extraction method consisting of a 2-step temperature program.

Hydrolysis

Solvent extraction is, undoubtedly, the most widely used procedure for the isolation of isoflavones from food samples. The direct analysis of extracts of isoflavones allows the native forms of isoflavones present in the sample to be determined; however, in many cases hydrolysis is still used, which reduces the number of chemical forms of isoflavones determined after sample treatment. Hydrolysis can be carried out before, during, or after extraction, and there are 3 procedures to achieve the hydrolysis of isoflavones: acidic, basic, or enzymatic hydrolysis. Acidic hydrolysis, the most commonly used method, breaks the glucoside bonds and transforms all the isoflavone derivatives into their aglycone forms. Alkaline hydrolysis breaks the ester bonds and removes acetyl or malonyl groups from the glucoside moiety, allowing just the β-glucosides and native forms of aglycones to remain after the procedure. Enzymatic hydrolysis produces the aglycones in a similar way to acidic hydrolysis.

Acidic hydrolysis generally involves treating the sample or the extract with alcoholic HCl within a concentration range of 1 to 6 M. For example, Csupor and others (2015) studied the effect of the concentration of HCl, hydrolysis time, and temperature on the total isoflavone aglycone content in dry soy extract containing products. The optimum hydrolysis conditions of an HCl concentration of 4.9 M, an extraction time of 96 min, and a temperature of 80 ºC were obtained by mathematical fitting and method optimization.

Boniglia and others (2009) analyzed the isoflavone content in soy-based dietary supplements after hydrolysis change to the corresponding aglycones, and carried out the extraction using a 3 M HCl ethanolic solution, sonication for 5 min, and by incubating the sample solution in a steam bath for 40 min. Clarke and others (2008) measured the concentration of isoflavones in dietary supplements. First, the samples were extracted with ethanol and ethanol–water, and sonicated for 5 and 30 min, respectively. The dried extracts were then hydrolyzed on a hot block at 95 ºC for 2 min, using 1 M aqueous HCl. After hydrolysis, the aglycones were extracted with diethyl ether. Alves and others (2010) added the antioxidant butylated hydroxytoluene (BHT) to the hydrolysis mixture, which was MeOH:3.4 M HCl (1:1, v/v) for ground coffee and MeOH:10.2 M HCl (1.7:0.67, v/v) for brewed coffee. The acid hydrolysis was performed at 75 ºC for 150 min, under reflux. Trifluoroacetic acid 2 M has also been used to carry out the acid hydrolysis of seed coats and hypocotyls of lupin, after an initial extraction with methanol (Ranilla and others 2009).

Klejdus and others (2008) determined aglycones and glucosides in plant material samples (*Trifolium pratense*, *Glycine max*, *Pisum sativum,* and *Ononis spinosa*) after acid hydrolysis with 2 M HCl and a 2-step temperature program Soxhlet extraction.

The AOAC Official Method 2001.10, described for the determination of isoflavones in soy and selected foods containing soy, includes alkaline hydrolysis. First, the samples are extracted at 65 ºC for 2 h in methanol:water (80:20, v/v) and then the extracts are saponified at room temperature with NaOH 2 M for 10 min. Before injection into the chromatographic system, the extracts are acidified using glacial acetic acid. Some modifications to the AOAC method included the use of acetonitrile instead of methanol, as proposed by Whent and others (2011), and use of ammonia instead of NaOH as the hydrolytic reagent, which has the advantage of eliminating any additional neutralization steps (Yuan and others 2008).

For enzymatic hydrolysis, β-glucosidase and glucuronidase are usually used. Fiechter and others (2010, 2013) performed the hydrolysis of isoflavones using β-glucuronidase from *Helix pomatia* juice, after methanolic extraction of the isoflavones. To adjust the concentration of MeOH to that of the initial chromatographic conditions, the methanolic extract containing isoflavones was diluted in 0.1 M sodium acetate buffer (pH 5) before the addition of the enzyme. Then the samples were incubated overnight (>12 h) using a thermal incubator at 37 °C and shaking at 200 rpm. Kuhnle and others (2007, 2009a, 2009b) employed a similar procedure, but they used a mixture of *Helix pomatia* juice (β-glucuronidase), cellulase, and β-glucosidase in sodium acetate (pH 5) as the hydrolysis reagent, and an incubation time of 16 h.

Shao and others (2011) evaluated 3 different hydrolysis methods and compared them with direct extraction, for the quantification of isoflavones in various soy products, and they found no significant difference among them. These authors also concluded that a maximum conversion of glucosides to aglycones occurred with acidic hydrolysis in 2 h; alkaline hydrolysis converted acetyl and malonylglucosides to their respective glucosides within 10 min and the β-glucuronidase of *H. pomatia* effectively converted glucosides and acetylglucosides to aglycones. Furthermore, another study comparing acidic and enzymatic hydrolysis was done by Konar and others (2012b). These authors compared the conventional extraction using MeOH:0.1 M HCl (5:1, v/v) for 2 h at room temperature, acid hydrolysis using 80% MeOH:3.4 M HCl (1:1, v/v) in the presence of BHT for 150 min at 75 ºC, enzymatic hydrolysis using cellulose, β-glucuronidase, and β-glucosidase solution at 37 ºC for 16 h, and a method combining both enzymatic and acidic hydrolysis. The study revealed that the enzymatic hydrolysis method was more effective for the identification of total isoflavone content. Similar results were obtained by Schwartz and Sontag (2009) who concluded that the isoflavone contents obtained by extraction and acid, basic or enzymatic hydrolysis, were similar.

Other extraction techniques

As mentioned above, several techniques have been developed over the last few years with the aim of achieving faster, more efficient, and very selective methods that use lower amounts of organic solvents.

Ultrasound-assisted extraction (UAE) is considered by some authors a modern technique of extraction, while for others it is a mode of solvent extraction where the use of ultrasound enhances the extraction efficiency. This improved efficiency is attributed to the phenomenon of cavitation produced in the solvent by the passage of an ultrasonic wave (Paniwnyk and others 2001). Ultrasound also exerts a mechanical effect, allowing greater penetration of the solvent into the matrix. Some authors have demonstrated that the use of high-power ultrasonication (HPU) resulted in a reduction in particle size of almost 10 times (Karki and others 2010). The reduction in particle size was directly related to sonication amplitude and the duration of exposure (Pananun and others 2012). As in conventional solvent extraction, the most influential parameters are the type of solvent, the temperature, and the duration of extraction. The most commonly used solvents in the ultrasound-assisted

extraction of isoflavones from food samples are, as discussed in the previous section, mixtures of MeOH, EtOH, or MeCN with water in different proportions (Table 1 and 2).

Microwave-assisted extraction (MAE) has also been used for the extraction of isoflavones from food matrices. MAE is based on the absorption of microwave energy (frequencies between 300 MHz and 300 GHz) by molecules of polar compounds. In this type of extraction, solvents with high dielectric constants are employed (water, MeOH, EtOH), since the energy absorbed is proportional to the constant. The main advantages of MAE are that less solvent is used, the extraction efficacy is enhanced, and MAE can be applied simultaneously to several samples, therefore reducing the time of extraction.

Rostagno and others (2007c) developed an analytical method for the extraction of 12 main isoflavones from soybean using MAE. These authors evaluated different extraction parameters: solvent type, solvent volume, temperature, and extraction time. The best results were obtained with 25 mL of 50 % aqueous EtOH at 50 ºC for 20 min. The comparison of the results with those obtained using UAE showed no significant differences for any isoflavone. In a similar way, Jiao and others (2012) used 90% aqueous ethanol at 50 ºC for 20 min for the extraction of isoflavones from different soybean varieties by MAE.

Careri and others (2007) optimized a microwave-assisted extraction method using a statistical data treatment based on experimental design for the extraction of genistein and daidzein from yellow soybeans. The extraction was carried out using MeCN:H_2O (80:20, v/v), the sample was sonicated for 15 min, and then, HCl 12 M was added before MAE was performed at 600 W for 1 min. The isoflavones of soy were determined as aglycones.

Terigar and others (2010) designed a continuous microwave-assisted extraction method to isolate isoflavones from soybean flour by connecting 3 microwaves in series using Teflon tubes. They optimized parameters such as temperature and extraction time, using ethanol as the solvent; the yield of extraction increased as the temperature increased and optimum values were obtained around 73 °C. Likewise, increasing the time of extraction also enhanced the amount of isoflavones extracted up until approximately 8 min; extraction times greater than 8 min did not produce significant differences. The authors concluded that the use of continuous microwave-assisted solvent extraction is a viable method at relatively short treatment times and high throughput.

There are several studies comparing microwave-assisted extraction with other techniques, such as Soxhlet, reflux, ultrasonic extraction, and stirring. However, MAE was more effective than all these other conventional techniques for the extraction of isoflavonoids (including formononetin) and saponins in Radix astragali (Song and others 2007). In this particular study, methanol was used as the solvent and the authors assessed the effect of microwave power and irradiation time on the extraction. Both parameters were shown to be closely related: at low microwave power (200 to 300 W) the extraction efficiency increased with irradiation time. However, the extraction yield decreased for irradiation times above 6 min, with a microwave power of 300 W, as well as for times greater than 4 min at 450 W. This may occur because the analytes decompose at high microwave power and long irradiation times. Also, Chukwumah and others (2007) showed that MAE and Soxtec methods extracted significantly higher amounts of isoflavones (genistein, daidzein, and biochanin A) and trans-resveratrol from peanuts. These results were attributed to the conversion of conjugate glycoside to the aglycones when methods involving heat treatment were used. These authors concluded that the choice of extraction method greatly depends on the characteristics of the analytes to be studied.

Pressurized liquid extraction (PLE) is a sample preparation technique involving the use of liquid solvents under high pressure (up to 200 atm) and temperature (up to 200 ºC) to improve the extraction efficiency of analytes from solid and semi-solid matrices. The use of elevated temperatures increases the solubility of the analytes, diffusion rates, and mass transfer, while high pressure keeps the solvent in the liquid state at temperatures above its boiling point. The main advantages of this technique are increased extraction efficiency, reduced use of solvents and extraction time, and the possibility of automation.

Several authors have compared this technique with other techniques commonly used for the extraction of isoflavones from food, such as conventional solvent extraction (CSE) and UAE. Luthria and others (2007) compared various commonly used extraction techniques (shaking, vortexing, sonication, stirring, Soxhlet, and PLE) for the extraction of isoflavones from soybean samples. These authors found that optimum recoveries of total isoflavones were obtained using PLE. The same results were observed by Zgórka (2009) when the PLE, UAE, and CSE extraction methods for isolating isoflavones from *Trifolium* species were compared. However, Lee and Lin (2007) showed higher extraction yields of puerarin, daidzin, and daidzein in Radix Puerariae when the ultrasonic technique was applied, as compared to PLE and CSE. These authors concluded that PLE was more suitable for the small-scale routine quantitative analysis of heat-stable components, while the ultrasonic method was applicable for the preparation of a large quantity of heat-labile herbal extracts.

The parameters that influence the efficiency of the extraction are type of solvent, temperature, pressure, sample amount, extraction time, and number of extraction cycles. Several authors have carried out studies on these variables for the extraction of isoflavones from different food matrices (Chang and Chang 2007; Zgórka 2009; Delgado-Zamarreño and others 2012a; Moras and others 2017). The solvent of choice for extraction of isoflavones is usually aqueous methanol or ethanol in percentages around 80%. Temperatures are higher than those used in conventional solvent extraction (>90 °C), while extraction times are somewhat shorter (5 to 15 min).

Zgórka (2009) studied temperatures of 75, 100, and 125 ºC, reporting the highest extraction efficiency at 125 ºC, although at higher temperatures no thermal degradation of the analytes was observed. Among the solvents tested (MeOH, acetone, and their 75% aqueous solutions), methanol:water (75:25, v/v) was chosen as it provided the highest PLE efficiency for the more polar compounds (De and Ge), and showed results comparable to those obtained using pure MeOH for the more hydrophobic isoflavones (For and Bio). In terms of time and the number of cycles, the best results were obtained using three 5-min cycles of static extraction. Chang and Chang (2007) examined the effect of pressure (413 to 4410 kPa), temperature (60 to 120 ºC), solvent flow rate (10 to 25 mL/min), ethanol:water ratio (0% to 95%), and feed loading (80 to 450 g) on the PLE of isoflavones and soya saponins from defatted soybean flakes, using the experimental design to optimize operational conditions. The best results were obtained using 80% ethanol extraction at 110 ºC, 551 kPa, and 25 mL/min with a 80 g feed loading. Delgado-Zamarreño and others (2012a) developed an analytical method based on pressurized liquid extraction (PLE) for the determination of isoflavones in Spanish pulses. In this study various variables were analyzed; the optimal extraction conditions were achieved using 2 to 3 g of sample, 90 °C, three 5-min

cycles of extraction and a maintained pressure of 110 atm. The most suitable extraction solvent varied depending on the type of sample. Thus, for chickpeas (where the major isoflavones are For and Bio) methanol/water (50:50, v/v) was the best solvent, while methanol/water (75:25, v/v) provided the best results for lentils (with Di, Gi, and For as the major isoflavones).

In addition, water can also be used in this technique (PWE), making it a more environmental-friendly procedure. This technique has recently been employed by Moras and others (2017) who studied the influence of the various operating conditions (temperature, contact time, liquid/solid ratio) on isoflavone extraction from soybean flour or soybean protein isolate (SPI) by pressurized water extraction using an experimental design. The extractions were carried out at a fixed pressure of 100 atm, using sand as the dispersing agent to avoid protein aggregation, and ultrapure water as the solvent. These authors demonstrated that the solid-liquid ratio used was the main factor influencing the extraction efficiency. The optimum extraction parameters were 120 ºC, 14 min, and 0.35 g of sample for soybean protein isolate, and 114 ºC, 2 min, and the same sample amount (which represented a 1/1, liquid/solid ratio) for soybean flour. The extraction yields in these conditions were 63.7% and 85.8 %, respectively. These low recoveries, compared with other proposed methods in which less polar solvent was used, can be attributed to the presence of aglycones in these matrices. Due to the weak polarity of these compounds, their extractability is lower with a polar solvent like water. This fact also explained the difference observed between the isoflavone extraction yield from soybean flour and SPI. The proportion of aglycon isoflavones was 21% in the SPI and less than 1% in soybean flour. The study also showed that malonyl isoflavones are transformed into glucosides between 80 and 160 ºC for both materials. At higher temperatures, the glucoside forms were converted into aglycones and the total isoflavone extraction yield decreased, since these forms were extracted with much lower yields. Therefore, the extraction efficiency of pressurized water depends to a large extent on the distribution of isoflavones, with PWE being limited to most polar conjugated forms (glucosides, malonyls).

Supercritical fluid extraction (SFE) has also been used to isolate isoflavones from solid matrices. This technique offers several advantages over conventional extraction methods, such as greater selectivity and reproducibility, higher speed, ease of automation, greatly reduced use of organic solvents, and improved environmental protection.

The most used supercritical fluid is carbon dioxide because it is chemically inert, nontoxic, nonflammable and low cost, and its critical parameters are easy to reach (31.1 ºC, 74.8 atm). Due to the nonpolar nature of carbon dioxide its application to the extraction of polar compounds is limited. However, the addition of a polar modifier (methanol, ethanol, water) is a simple and effective way of modifying the polarity of the supercritical fluid to enhance the solubility of polar analytes.

In the extraction of isoflavones, SFE has been used by several authors, in all cases in the presence of modifiers. Bajer and others (2007) compared different extraction techniques (supercritical fluid extraction, pressurized fluid extraction, matrix solid-phase dispersion, ultrasonic extraction in an ultrasonic bath, and by means of an ultrasonic homogenizer, Soxhlet extraction, and solid-phase extraction) for the extraction of isoflavonoids from leaves of several plant species. The authors concluded that it is impossible to suggest a single method that ensures all isoflavonoids are extracted and with maximum yields, since the individual isoflavonoids studied (daidzein, genistein, apigenin, and biochanin A) can be extracted with maximum yields using different methods. Thus, De and Ge had the greatest extraction yields using SFE, Soxhlet, or ultrasonic techniques, while for Bio and apigenin the best results were obtained using SFE. In addition, SFE offered other advantages, such as less solvent use and shorter extraction time, and it produced sufficiently pure extract without the need for subsequent filtration (similar to PLE).

Araújo and others (2007) used SFE to extract isoflavone aglycones (De and Ge) from soybean hypocotyls after enzymatic hydrolysis of the glucosidic isoflavones with β-glucosidases. The highest yields of isoflavones were obtained at 60 ºC, 380 bar using 10 mol% of 80% MeCN, and static or dynamic extraction for 15 min. The authors also compared the extraction efficiencies with those obtained by conventional solid–liquid extraction using 80% aqueous methanol, which were higher than those attained by SFE.

Kao and others (2008) used 70% ethanol as the modifier for the extraction of 12 main isoflavones from soybean cake, and the authors evaluated the influence of temperature and pressure. Theoretically, higher pressures increase extraction efficiency; however, a high pressure may also decrease supercritical fluid diffusivity leading to a lower extraction yield. On the other hand, at low pressure, the extraction efficiency increased as the temperature increased. The authors also showed a higher yield of malonylglucosides and glucosides at 60 ºC and 350 bar, while a high amount of acetylglucosides and aglycones were produced at 80 ºC and 350 bar.

In comparison with solvent extraction, higher yields of malonylglucosides and glucosides were obtained with solvent extraction, whereas for acetylglucoside and aglycones greater contents were obtained by SFE (agree with nonpolar nature of supercritical carbon dioxide). In general, solvent extraction produced a much higher yield of total isoflavone than supercritical carbon dioxide extraction. The article also shows that isoflavone conversion or degradation can still occur when in combination with pressure.

Another report describing the influence of the different extraction parameters was published by Zuo and others (2008). The analysis of the composition of the modifier showed that isoflavone recovery was greater as the water content in the methanol increased up to 20%, which was due to the increased polarity of the modifier. Higher water concentrations were not suitable for isoflavone extraction. The authors also showed that modifier concentrations up to 10.2 mass% increased the recovery of isoflavones. The increase in temperature resulted in a decrease of the extraction yield, due to reduction of the solvent density which reduced the solvent power of the fluid. The highest isoflavones recovery was achieved at 40 ºC and 50 MPa, with a CO_2 flow rate of 9.80 kg/h.

Although several authors have verified the increase in extraction yield under pressure, Klejdus and others (2010) demonstrated that it depends on the type of analyte. In addition, they showed that the best extraction pressure was 35 MPa for Di, Gly, De, and For; 30 MPa for Gle and Ge; and 40 MPa for Gi, Bio, ononin, and sissotrin. According to these authors, the optimum extraction pressure for each compound was the consequence of the maximum positive effects (mass transfer, analyte solubility) and the minimum negative effects (like restrictor plugging). The latter effect occurrs because a change in the pressure used during the extraction could change the amount of modifier that is soluble in the extraction phase. If some component of the modifier was not soluble in the supercritical phase and becomes a liquid, or the whole fluid drops from supercritical to near-critical state, the extraction fluid would have less solvation power. In addition, liquid drops can

plug the restrictor, decrease the flow-rate of the extraction fluid, and therefore lead to lower amounts of the analyte being carried out from the extraction cell. The authors also concluded that the matrix effect seems to influence the extraction pressure and temperature, but the best amount and composition of the modifier seem to depend more on the type of analyte and less on the matrix. The proposed method for the determination of isoflavones in algae included sonication of the sample, followed by extraction with supercritical CO_2. The optimum extraction conditions were: 35 MPa, 40 ºC, 60 min, and 3% (v/v) MeOH:H_2O (9:1, v/v) modifier.

Anastassiades and others (2003) developed a new method for the extraction of a broad range of pesticide residues from fruits and vegetables. This method was called *QuEChERS* (acronym of Quick, Easy, Cheap, Effective, Rugged, and Safe), which refers to the main advantages of the method, as compared to the traditional method which involved multiple stages and large sample amounts. This method has been modified according to the properties of the analyte, the matrix composition, and the techniques and equipment available in the laboratory (Lehotay and others 2010). Recently, the QuEChERS method has been applied to the determination of isoflavones in legumes (Delgado-Zamarreño and others 2012b, Bustamante-Rangel and others 2014), soy-based foods (Bustamante-Rangel and others 2013), and soybeans (Ding and others 2016).

Delgado-Zamarreño and others (2012b) proposed, for the first time, a method based on QuEChERS for the extraction of isoflavones, substances naturally present in food samples, unlike the original method designed for exogenous compounds. The target compounds were the glucosides Di, Gly, and Gi, and the aglycones De, Ge, Gle, For, and Bio. In order to optimize the extraction conditions, different mixtures of MeCN, MeOH, and EtOH with water in different proportions were investigated. In view of the different polarities of the analytes studied, the authors proposed a 2-step extraction process. They found greater extraction efficiency for all of the analytes analyzed when a 2-step extraction process was used; first, the more polar analytes were extracted, and then the less polar analytes were extracted by decreasing the polarity of the solvent. Hence, the extraction of the analytes from the legume samples was done by adding MeCN:H_2O (70:30, v/v) and by vortexing for 5 min. Then pure MeCN was added and the mixture was vortexed for another 5 min, achieving a final proportion of MeCN:H_2O (80:20, v/v). Finally, a mixture of $MgSO_4$:NaCl (4:1, w/w) was added and vigorously vortexed for 1 min to prevent the formation of conglomerates prior to centrifugation. The authors also proved that the developed procedure allowed the final cleaning step by dispersive-solid-phase extraction (d-SPE) to be eliminated. This method was successfully applied for the determination of isoflavones, including aglycones and glucosides, in legumes of Spanish origin (chickpeas, lentils, and white beans). These authors (Bustamante-Rangel and others 2014) also verified that the determination of isoflavones in foods can be affected by the different methods used to place the sample and the solvent in contact during the extraction stage. To do this, they compared different methods for placing the sample and the solvent in contact during the extraction of isoflavones from legumes using the previously developed QuEChERS method. Five different approaches for mixing were evaluated: vortex agitation, 2 mix-stirring methods (thermostatted stirring agitation and thermostatted tray shaking), and 2 ultrasound-based-assisted extraction (thermostatted ultrasound bath and ultrasound probe). It was found that the thermostatted shaking tray produced the best results for chickpeas and white beans and the ultrasound probe for lentils.

Additionally, Bustamante-Rangel and others (2013) also developed a QuEChERS approach for the extraction of isoflavones from solid soy-based products (biscuits). The sample was pretreated with hexane in order to remove some of the fat from the samples. The defatted samples were then subjected to the QuEChERS method using MeCN:H_2O (50:50, v/v) as the extraction solvent. The samples were shaken for 5 min using an automatic inversion mixer at room temperature. Following this, a mixture of $MgSO_4$:NaCl (4:1, w/w) together with a mixture of 1 g of Na_3citrate.2 H_2O and 0.5 g of Na_2Hcitrate.1.5 H_2O (citrate buffer) were added. Then, the tube was shaken vigorously for 1 min to prevent the formation of $MgSO_4$ conglomerates, and centrifuged. A portion of the extract (1.0 mL) was subjected to d-SPE using a mixture of 150 mg of $MgSO_4$, 150 mg of silica sorbent, 25 mg of PSA, and 25 mg of C18 sorbent. The signals increased and the stability improved when the cleaning step was used with a mixture of the 3 sorbents. The authors attributed these results to the fact that the interferences extracted from the sample, such as fatty acids, triglycerides, and phospholipids had greater affinity for C18 and silica sorbents, while other extracted compounds, such as carbohydrates or pigments, exhibited a higher affinity for PSA.

More recently, Ding and others (2016) have applied a modified version of the QuEChERS method in combination with UAE for the extraction of 6 isoflavones (Di, Gly, Gi, De, Ge, and Gle) from genetically modified and nongenetically modified soybeans. The extraction conditions were optimized by applying the orthogonal experiment and response surface method designs. The optimum conditions for the extraction were achieved with MeCN:H_2O (70:30, v/v) as the extraction solvent, $MgSO_4$:NaCl (4:1, w/w), and 20 min of sonication.

Among the procedures developed to achieve faster, more cost-effective and environmentally friendly sample treatment methods, *matrix solid-phase dispersion* (MSPD) should be considered. This technique, introduced by Barker and others (1989, 2000), combines the simultaneous disruption and extraction of solid and semi-solid matrices. To do this, disrupted samples are dispersed on and into a solid support (C18, Florisil, aluminum oxide, silica gel), thereby generating chromatographic material that possesses a unique characteristic for target analyte isolation. This approach requires very small solvent volumes, a small amount of sample, a short extraction time, and often combines extraction and clean-up into 1 single step.

MSPD has been used by Barfi and others (2009) for the extraction of daidzein and genistein in soybean and its waste. To optimize the extraction method, the type of sorbent, the sorbent to sample ratio and the type, volume, and pH of the elution solvent were evaluated. Two sorbents (aluminum oxide and silica gel) and different solvents (methanol, acetonitrile, diethyl ether, ethyl acetate, dichloromethane, and n-hexane) were examined. The best recoveries of both of the analytes studied were achieved by using methanol at pH 3, silica gel as the sorbent, and a 2:1 (w/w) ratio of silica gel to soybean. Prior to analyte elution, the column was washed with n-hexane to remove interfering compounds.

Visnevschi-Necrasov and others (2009) optimized a matrix solid-phase dispersion extraction method for the analysis of isoflavones in red clover leaves. They found that the type of the sorbent and the extraction solvent polarity are the most important factors in MSPD extraction. The selected sorbent was C18, and the elution solvent was dichloromethane:methanol (25:75, v/v). They also tested an additional clean-up step using sorbents such as

GCB, HLB, and mixtures of $MgSO_4$, C18, and PSA. However, these sorbents produced lower recovery values for the isoflavones studied than the extraction procedure without the clean-up step. Since the remaining co-extracts after extraction did not negatively affect the resolution or the column life during the time of validation, the clean-up step was not included. These authors have also developed MSPD-based procedures for the extraction of isoflavones from different *Medicago* species (Barreira and others 2015; Visnevschi-Necrasov and others 2015) and other plants belonging to the *Leguminosae* family (Cunha and others 2012), with the aim to evaluate isoflavone profiles. In this case C18 was used in a sorbent:to:sample ratio of 4:1 (w/w). The isoflavones were eluted with MeOH:H_2O (9:1, v/v).

One of the most commonly used extraction techniques for liquid samples is *solid phase extraction* (SPE). However, it should be noted that this technique has only been used on very few occasions for the extraction of this type of analytes. For example, Zhao and others (2015) have used a mixed-mode SPE coupled with liquid chromatography tandem mass spectrometry for the simultaneous determination of isoflavones and resveratrols for adulteration detection of soybean and peanut oils. These authors used a mixed-mode weak ion exchanger. The cartridges were previously conditioned with methanol and n-hexane and dried with a stream of nitrogen. Then, the sample previously diluted with n-hexane was percolated through the cartridge. The optimized washing solvent was isopropyl alcohol/n-hexane (60:40, v/v); this solvent mixture was found to be suitable for retention of target analytes and to remove lipophilic matrix compounds. Finally, methanol was used for analyte elution, taking into account their polarity. However, SPE is mainly used for cleaning and preconcentration of extracts after solvent extraction of the analytes from the samples, as can be seen in Table 1 and 2.

An alternative method to SPE is *solid phase micro extraction* (SPME), which involves the use of a coated fused-silica fiber that allows for processes of mass transfer. This technique integrates all the phases of the process of sample preparation (extraction, concentration, and sample introduction) into 1 step and 1 device. SPME is rapid, solventless, and readily adaptable for automation extraction technique. In isoflavone extraction, this technique has been employed by Aresta and others (2016) for the determination of daidzein, genistein, glycitein, genistin, and equol in soy drinks. To optimize the extraction, the authors evaluated the variables that affected both adsorption and desorption of the analytes. Three different types of fiber coating were tested: polydimethylsiloxane (PDMS), polydimethylsiloxane–divinylbenzene (PDMS–DVB), and polyacrylate (PA). Although all of the fibers were able to extract the analytes, PDMS-DVB yielded the most efficient extraction. The fiber was exposed to the solution under constant magnetic stirring for 20 min at room temperature. Under these nonequilibrium conditions, good extraction yields, and reliable analysis were achieved. Compound desorption was performed in static mode by soaking the fiber in mobile phase directly in the desorption chamber of the interface of the chromatograph for 15 min. A notable positive effect on extraction was observed in the presence of the acidic medium and the addition of salt, except for equol. As a result, the samples were diluted 1:10 with 0.2% formic acid at 30% (w/v) NaCl.

In recent years, a new type of SPE based on the use of magnetic sorbents has been developed. This extraction technique, called *magnetic solid-phase extraction* (MSPE), has now been studied because of its potential applications in several fields of separation science. Generally, the sorbent material does not need to be packed in any type of device, as in traditional SPE (Vasconcelos and Fernandes 2017). This technique requires small volumes of sample and solvent for extraction and desorption, concentrates the analytes, and produces high recovery owing to the large surface area provided by the dispersion of the sorbent into the matrix. In addition, the phase separation can be easily achieved by using an external magnet placed outside the extraction vessel without the need for centrifugation or filtration. Magnetic particles are available in a wide range of sizes (from nanoscale to microparticles). The particle surface is usually coated with inorganic materials, such as silica, alumina, manganese oxide, and graphene, or organic compounds such as polypyrrole, molecularly imprinted polymers (MIP), chitosan, and surfactants. Moreover, the addition of a suitable functional group can improve the sorption properties of the material.

This technique has been used by Qing and others (2013) for the determination of isoflavones in soymilk. These authors employed baicalin-functionalized core-shell magnetic nanoparticles to selectively extract isoflavones from soymilk without involving a freeze-drying procedure for subsequent HPLC–ESI-MS/MS analysis. The selective extraction of isoflavones was achieved by means of the molecular affinity between baicalin in the nanoparticle and isoflavones because of its common skeleton. Due to the skeleton (C6-C3-C6 π-conjugated system), baicalin anchored on the magnetic particles can be simply prepared for selective and efficient separation and enrichment of isoflavones, without template molecule removal. A similar approach was used by Guo and others (2017) for the extraction of soymilk isoflavones, using chitosan, a natural polymer material, in combination with molecularly imprinted technique. They used rhein anchored on magnetic chitosan microparticles as sorbents for the extraction of target analytes. After completion of the extraction process, Fe_3O_4 particles acted as carrier to retrieve rhein-functionalized magnetic chitosan microparticles from the sample solution.

Other approaches for the extraction of isoflavones have included the use of supramolecular solvents (Magiera and others 2016), reverse micelles (Zhao and others 2010; Mirzaei and others 2012; Cordisco and others 2016) or ionic liquids (Sun and others 2011; Magiera and Sobik 2017).

Supramolecular solvents (SUPRASs) are nanostructured liquids made up of 3-dimensional aggregates of amphiphilic compounds. SUPRASs are generated through self-assembly processes, induced by changes in the environmental conditions of aqueous or hydroorganic solutions of the amphiphile (for example, pH modification, salt addition, presence of a nonsolvent for the amphiphile, and so on). Self-assembly causes the spontaneous separation of an amphiphile-rich liquid phase from the bulk solution. One unique property of SUPRAS is the presence of different polarity regions in their constituents and hence their ability to dissolve, concentrate, compartmentalize, organize, and localize solutes spanning wide polarity ranges, thus providing specific reaction environments and separation media. Due to the ability of SUPRAS to extract analytes in a wide polarity range and the high extraction efficiency they provide, these solvents have great potential for developing sample handling approaches. SUPRASs made up of reverse micelles of decanoic acid dispersed in tetrahydrofuran-water have been used by Magiera and others (2016) for the extraction of isoflavones (De, Ge, Bio, and Gly) from different soy foods (soybeans, soy flour, breakfast cereals, soy drink, and others).

Reverse micelles have been employed in the extraction of isoflavones since hydrophilic isoflavones tend to be solubilized within the water core of the reversed micelles, while lipophilic

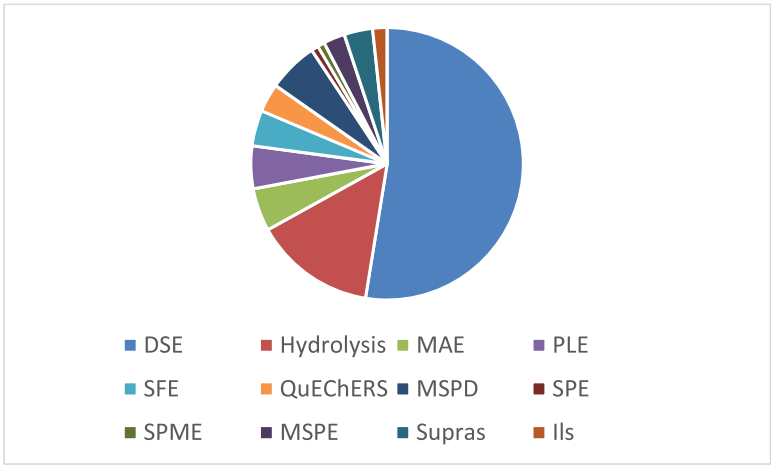

Figure 2–Comparison among the sample treatments applied to the analysis of isoflavones.

isoflavones can either stay in the interface or be partially exposed to the organic phase. Different reverse micelles of anionic surfactants sodium bis(2-ethyl hexyl) sulfosuccinate (AOT) and sodium dodecyl sulfate (SDS), cationic surfactant hexadecyl trimethyl ammonium bromide (CTAB) and nonionic surfactant polyoxyethylene p-t-octylphenol (TritonX-100) in organic solvent isooctane were investigated for the extraction of isoflavones from soybean flour samples by Zhao and others (2010). Ethylene glycol monoalkyl ether (genapol X-080) has also been used as nonionic surfactant in the extraction of genistein from soybeans (Mirzaei and others 2012). Aqueous micellar systems formed by polyethylene glycol tert-octylphenyl ether (Triton X-114) has been used to extract and purify soy isoflavones (Cordisco and others 2016).

Among the trends toward eco-friendly analytical methods, *ionic liquids* (ILs) are gaining widespread recognition as novel solvents. ILs generally consist of bulky, nonsymmetrical organic cations such as imidazolium, pyrrolidinium, pyridinium, ammonium, or phosphonium and numerous different inorganic or organic anions such as tetrafluoroborate and bromide anions. ILs have many attractive physicochemical properties, such as chemical and thermal stability, nonflammability, high conductivity, in many cases miscibility with water and organic solvents, and good solubility of various compounds. Ionic liquids are considered as "green" solvents because of extremely low volatility. Furthermore, their polarity, hydrophobicity, viscosity, and solvent miscibility can be selected by choosing the cationic or the anionic constituent. ILs are regarded as "designer solvents" because of this modifiable nature, which increases their potential applications. Ionic liquids have recently been used in the extraction of isoflavones from soy products by Magiera and Sobik (2017). These authors developed an ionic liquid-based ultrasound-assisted extraction (ILUAE) method to extract 4 isoflavones (Gi, Ge, Di, and De). They studied the type of anion, aliphatic chain length, IL concentration, solid/liquid ratio, and extraction time to obtain the best extraction efficiency using a central composite design. 1-hexyl-3-methylimidazolium bromide ($[C_6MIM]Br$) was chosen as ionic liquid, at a concentration 1M. An ILUAE method has been also employed by Sun and others (2011) for the extraction of 3 isoflavones, namely tectoridin, iristectorin B, and iristectorin A from *Iris tectorum* Maxim, a very popular Chinese traditional medicinal herb belonging to the Iridaceae family.

As can be seen in Figure 2, more than 50% of the extraction methods applied during the last few years for the analysis of isoflavones involve direct solvent extraction (DSE), and almost 70% involve conventional extraction methods. However, techniques such as PLE, MAE, SFE, or SPE provide very significant advantages compared to these conventional techniques. In addition to the afore-mentioned advantages, selectivity, efficiency, reduction in organic solvent volume, and time, these techniques require less sample handling, which also reduces the errors associated with the method.

Separation and Detection Techniques

Several analytical methods have been used for the separation and detection of isoflavones such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), gas chromatography (GC), and immunoassay. Among these, reversed-phase liquid chromatography with spectrophotometric detection is the most widely used technique for separation and quantification of isoflavones from foods. This technique allows the determination of isoflavones without derivatization, unlike GC, in their natural form (aglycones or glycosides) and provides high efficiency, sensitivity, speed, and can be used in automated systems.

Thin-layer chromatography

Thin layer chromatography (TLC) is used practically for preparative purposes (Zhao and others 2009), as is centrifugal partition chromatography (CPC) (Jeon and others 2014). High-performance thin-layer chromatography (HPTLC) has been used for quantification of the glycosidic isoflavones Di, Gi, and Gly in soybean (Puri and Panda 2015). Chromatographic separation was performed on aluminum foil-backed silica gel 60 F254 HPTLC with toluene:ethyl acetate:formic acid:acetic acid (1:8:1:0.5, v/v/v/v) as mobile phase and densitometric UV detection at 260 nm.

Gas chromatography

Gas chromatography (GC) has been widely used in phytoestrogen analysis, mainly in biological fluids (Šošić-Jurjević and others 2014, 2017), due to its high resolution, selectivity, and sensitivity. However, since the low volatility of these compounds, the procedure requires the sample to be previously derivatized, which makes

the sample treatment step longer and tedious; furthermore, this additional step could introduce errors in the final results due to analyte losses. Some papers regarding the determination of isoflavones by GC-MS have been published, mainly involving fragmentation studies for the elucidation of structures (Maul and others 2008; Ferrer and others 2009).

Capillary electrophoresis

Despite the inherent advantages of capillary electrophoresis (CE), such as high separation resolution, efficiency, low sample and reagent consumption, and short analysis time, this technique has not been widely used for the determination of isoflavones in foods. Several CE techniques have been developed, including capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MECC), and capillary electrochromatography (CEC). Since most phytoestrogens and their metabolites contain phenolic hydroxyl groups and have a weak acidic nature, CZE methods are generally performed based on a borate or acetate buffer run at alkaline pH to ensure the analytes will be present as anions for electrophoretic separation. In general, by increasing the pH of the buffer, the resolution of the separation improved. In some cases, organic solvents such as methanol or acetonitrile were used as modifiers to improve the separation resolution, although this usually increases the migration times. With the commonly used separation buffers, formononetin and biochanin A, which have very similar chemical structures, could not be separated. Xiao and others (2015b) have investigated the use of ionic liquids as additives to improve separation of these isoflavones. They found that the addition of ILs had little effect on the EOF, suggesting that the separation mechanism is most likely to be either hydrophobic interactions between the analytes and the ILs and/or the formation of complexes between the analytes and the IL. Using 1-butyl-3-methylimidazolium tetrafluoroborate ($BMImBF_4$), they observed that the migration time of the compounds with high log *P* values decreased with increasing concentrations of the IL, which may be associated with the hydrophobic interaction of the analytes with the hydrophobic cationic imidazolium of $BMImBF_4$. Furthermore, the migration time of analytes with higher log P values (such as biochanina A) decreased much more than that of formononetin (with lower log P), which allowed the separation of these analytes.

Micellar electrokinetic capillary chromatography (MECC) has also been used to separate isoflavones. Accordingly, Zhang and others (2007) developed a MECC method for the determination of Bio, For, Ge, and De in red clover using an electrolyte composed of 30 mM borate, 20 mM sodium dodecyl sulfate (SDS), and hydroxypropyl-β-cyclodextrin (HP-β-CD) containing 5% (v/v) ethanol at pH 10.1. They achieved the separation of the 4 isoflavones in a shorter time and with a better resolution than other previously published methods. Similar conditions (10 mM sodium borate at pH 9.4 containing 15 mM SDS and 5% (v/v) methanol as running buffer) were used by Xiao and others (2011) for the determination of 6 isoflavones (De, Di, Ge, Gi, Gle, and Gly) in soybean meal and fermented soybean meal. Xiao and others (2015a) compared different micellar systems: single surfactants -anionic (SDS and a ionic liquid-type surfactant), cationic (CTAB) and neutral (polyoxyethylene sorbitan monolaurate, Tween 20)-, different single surfactants with $BMImBF_4$ as IL modifier, and mixed micelles of SDS. The best separations were achieved by using of SDS micelles with IL additive and mixed micelles of SDS/Tween 20. The first was chosen because they produced shorter analysis times and was a more stable buffer system, which resulted in good repeatability of retention time and peak shape of analytes.

Detection in CE is usually performed with UV (Dinelli and others 2007; Xiao and others 2015b) and electrochemical detection (ED) (Chen and others 2008). Mass spectrometry (MS) coupled to the EC is a powerful alternative combining the cited advantages presented by the EC, with the selectivity of the analysis and the structural information provided by the MS. This combination provides a second dimension to the separation, since the analytes are not only separated by their charge/size ratio (as in the case of CZE) but also in terms of their mass/charge ratio (m/z). In spite of the advances and the increase of applications that have occurred in the last decade, the applications of CE-MS in quantitative analysis are still limited. Bustamante-Rangel and others (2012, 2013, Pérez-Martín and others 2015) applied capillary zone electrophoresis coupled with electrospray ionization mass spectrometry (CZE–ESI-MS) for the separation and quantification of isoflavones (aglycones and glucosides) in soy products (soy drinks and soy biscuits) and legumes. García-Villalba and others (2008) used capillary electrophoresis time-of-flight mass spectrometry to compare the metabolic profiles of conventional and genetically modified soybeans, including isoflavones, amino acids, carboxylic acids and peptides.

Liquid chromatography

Liquid chromatography (LC) is the most commonly used technique in the determination of bioactive compounds in food. Compared to GC, HPLC offers the advantage of operating at lower column temperatures and without the need to derivatize the analytes. In addition, it allows the determination of all the chemical forms of isoflavones, requires simple preparation of the sample, produces highly efficient and reproducible separations, and has a great ruggedness. Also, the majority of LC separations of isoflavones were carried out by reversed phase, using octadecyl-silica (C18) phases. However, the use of conventional C18 (5 μm) columns leads to long analysis times. Mobile phases are usually mixtures of MeOH or MeCN with water, containing a small amount of acid (acetic, formic, trifluoroacetic) which produce enhanced chromatographic separation and improved peak shape. Since the chemical forms of isoflavones are very similar, chromatographic separation of all of them usually requires performing a gradient elution. A large number of the references already mentioned in this review use liquid chromatography for the determination of isoflavones, and they are shown in Table 1 and 2.

Current trends in liquid chromatographic analysis involve the development of reliable, fast, efficient, and sensitive methods. The main modern approaches involving HPLC methods, which enable the reduction of analytical time without compromising resolution and separation efficiency, use monolithic and fused core columns, liquid chromatography at high temperatures and liquid chromatography at ultra-high pressures using sub-2-microne-particle packed columns (Nováková and Vlcková 2009).

One of the first approaches has been the use of high flow rate and/or short column. However, the use of high flow rates increased the back pressure, leading to a decrease in the theoretical plate number, as with the use of short columns. The reduction of the particle size of the stationary phase has been also widely used (Toro-Funes and others 2012, 2014a, 2014b), which resulted in shorter analysis times with high sensitivity and separation efficiency. The ultra-high-pressure liquid chromatography (UHPLC) has made it possible to achieve 5- to 10-fold faster separations. The use of sub-2-μm-particle packed columns also leads to an

increase in the back pressure, which has led to change the currently available equipment to high-pressure-resistant models for analysis. Klejdus and others (2008) have achieved complete separation of 10 isoflavones (including aglycones and glucosides) in 1.5 min using UHPLC with different stationary phases (nonpolar reversed phase C18 and more polar phases with cyanopropyl or phenyl groups) with a particle size under 2 μm.

Monolithics have been introduced as a new separation material to obtain a high theoretical plate number without increasing the back pressure on the column. The use of such columns is expected to allow high-speed separation with high performance, since monoliths can accept high flow rates (up to 10 mL per min) at conventional column lengths without generating high return pressures, which is their main advantage. The main monolithic types are those based of functionalized silica derived from sol–gel synthetic routes and those based on cross-linked macroporous polymer networks derived from free-radical polymerizations. Two monolithic columns have been used in the analysis of 12 main isoflavones by Rostagno and others (2007a). Perhaps the main drawback of monolithic columns is their low robustness.

Manchón and others (2011) have compared 3 different chromatographic columns for the analysis of main soy isoflavones: a conventional reversed-phase (3.5 μm) particle column, a fused-core (2.6 μm) particle column, and a monolithic column. The comparison was made in terms of chromatographic parameters such as resolution, asymmetry, number of theoretical plates, variability of retention time, and peak width. The authors also evaluated the effect of sample solvent on the separation and peak shape. The conventional particle column revealed good chromatographic performance, but relatively long analysis time. Monolithic column produced the lower pressures, which allowed increasing the flow rate and decreasing the analysis time; although lower chromatographic performance was achieved. Moreover, it is the columns which was most affected by higher concentrations of organic solvents. Fused-core column showed the best chromatographic performance and separation speed.

Liquid chromatography has been coupled with a wide range of detectors, including UV-visible, diode array, fluorescence, electrochemical (Matsumoto and others 2010; Popa and Diculescu 2013), nuclear magnetic resonance (Yerramsetty and others 2011; Bórquez and others 2013), and mass spectrometry. Nowadays, the most common used detectors are UV, DAD and MS.

Spectrophotometric detection has been the method of choice for a long time because all structures of the isoflavones show a characteristic UV spectrum due to the conjugated systems of the aromatic rings. The absorption spectrum of most isoflavones consists of 2 maxima, one in the range of 240 to 285 nm (Band II, originated from ring A or benzoyl system) and the other in the range of 300 to 400 nm (Band I, associated to ring B or cinnamoyl system). Therefore, UV detection of isoflavones is normally carried out at 254 to 260 nm, while for DAD monitoring in the characteristic range of Band II is performed. It should be noted that, although UV–Vis spectra can be used to distinguish between different classes of flavonoids, it does not allow differentiation between different isoflavones, since their UV-Vis spectra are very similar. MS detectors are able to detect individual aglycones, glucosides, and other conjugates, and they have become a powerful tool for the identification and quantification of isoflavones from foods. In addition, tandem MS can be used for structural elucidation of the structures of isoflavones. The most widely used ionization sources used in the analysis of isoflavones are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Matrix-assisted laser desorption and thermospray ionization are used less frequently.

Conclusions

The nutritional and bioactive properties of foods are under constant surveillance. This has led to a growing interest in developing faster, more selective, and better sensitive methods of analysis. This work focuses on the methods used in the last 10 years for sample preparation, separation, and detection of isoflavones in food samples. The most commonly used procedure for the extraction of analytes from food samples is direct solvent extraction. In the case of isoflavones, the most commonly used solvent is methanol, mixed in different proportions with water, although it has been shown that MeCN:water mixtures also produced good results, especially for the extraction of the glucosides and aglycones. Extraction is usually achieved by stirring, shaking, vortexing, or sonication. Other techniques such as MAE, PLE, SFE, or SPE have been also applied with the aim of achieving faster, environmentally friendly, more efficient, and highly selective methods. Moreover, these techniques require less sample manipulation, which reduces errors in the determinations. The most current trends in this regard are the use of supramolecular solvents and ionic liquids. Within the separation and detection techniques, liquid chromatography coupled to spectrophotometric detection is undoubtedly the most commonly used method, although mass spectrometry is increasingly being used. Current trends in liquid chromatographic analysis involve the use of monolithic and fused-core columns, liquid chromatography at high temperatures and liquid chromatography at ultra-high pressures.

Acknowledgments

The authors acknowledge financial support from Univ. of Salamanca "Programa propio de Financiación de Grupos de Investigación."

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