SPECIAL GUEST EDITOR SECTION

Analysis of Isoflavones in Foods and Dietary Supplements

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Isoflavones are phytochemicals found in many plants. Because of their structural similarity to β-estradiol, health benefits of isoflavones have been evaluated in age-related and hormone-dependent diseases. Daidzein, genistein, and glycitein are present as free forms or derivatives in foods containing soy or soy protein extracts. The analysis of isoflavones has become more complex, because preparations contain isoflavones from multiple sources (e.g., red clover, kudzu). Red clover contains primarily formononetin and biochanin A, while kudzu extracts, which are becoming increasingly common in dietary supplements, contain puerarin and daidzein, among other components. Isoflavones are present in foods and dietary supplements as free compounds, glucoside derivatives, 6″-O-malonyl-7-O-β-D-glucoside derivatives, and 6″-O-acetyl-7-O-β-D-glucoside derivatives. High-performance liquid chromatography (HPLC)/tandem mass spectrometry has been applied to the identification of isoflavone derivatives based on the fragmentation pattern of the parent ion, providing high selectivity and sensitivity in the quantitation of isoflavones in complex mixtures. HPLC with ultraviolet detection is often chosen for routine analysis, but a preliminary acid or basic hydrolysis of isoflavone derivatives is often required for the investigation of samples containing extracts from multiple sources. Several internal standards have been used in the analysis of isoflavones from a single botanical source (e.g., soy, red clover), but the identification of a general internal standard remains a challenging process.

Isoflavones are phytochemicals that are widely distributed in nature. Seeds and other parts of many plant species belonging to the *Leguminosae* family have been found to contain isoflavones. Health effects of isoflavones, mainly related to their structural similarity to β-estradiol, are described by other authors elsewhere in this *Special Guest Editor Section*. Over the last decade, isoflavones have been extensively studied, and a large number of original research and review articles have been published (1, 2). Several methods for the analysis of isoflavones from specific sources, such as soy, soy-containing foods, kudzu, or red clover extracts, have been described (3–6). Wang et al. (1) described the analytical techniques applied to the quantitation of isoflavones, while Merken and Beecher (2) summarized high-performance liquid chromatography (HPLC) conditions applied to the analysis of isoflavones from different matrixes. Earlier publications have been dedicated to the general analysis of phytoestrogens or polyphenolic compounds (1, 2), while recent publications were dedicated to the analysis of isoflavones (3–8). To date and to our knowledge, the only method for quantitation of isoflavones that has undergone a collaborative study is AOAC Official Method **2001.10** (3); this method is limited to the analysis of isoflavones in soy or soy-containing foods. The majority of the methods reported in the literature have been optimized for the analysis of isoflavones in specific products and are not appropriate for the analysis of isoflavones in different matrixes. To date, there is not a general method for routine analysis of mixtures of isoflavones from different sources that has undergone a collaborative study.

Isoflavones—Chemistry and Sources

The distinctive core of isoflavones is a 3-phenyl-chromen-4-one conjugated system, and single isoflavones are characterized by the presence of substituents such as methoxy, hydroxyl, and glycoside functions on the primary structure. Figure 1 shows the structure of the main isoflavones that have been reported in foods and which are commercially available as reference materials. Isoflavones are usually present in foods and plant extracts as glycoside derivatives. The 7-O-β-D-glucosides are the most common isoflavone derivatives. Some plants produce derivatives such as puerarin (8-C-β-D-glucosyl daidzein) in which the sugar is linked to the isoflavone with a C-C bond. The different bonds between sugar and isoflavone affect the chemical properties of these compounds, including the efficacy of acid sample digestion. Diglucoside derivatives of isoflavones are less common and are found in the extracts of plants such as *Pueraria lobata* (kudzu). 6″-O-malonyl-7-O-β-D-glucoside derivatives are the predominant form of isoflavones in

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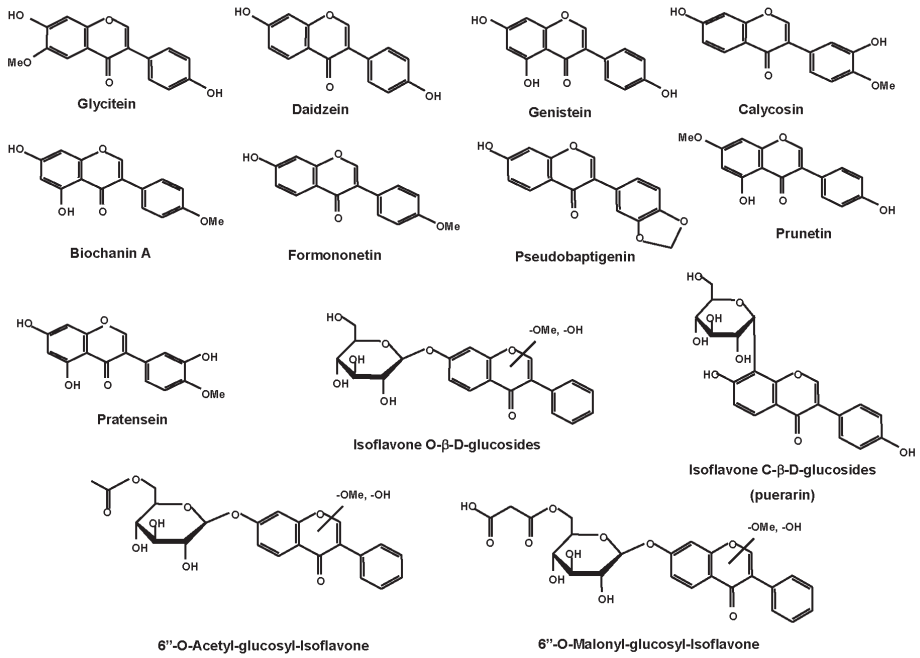

Figure 1. Structures of the primary isoflavones reported in food and plants.

plants (5–8). These derivatives are not chemically stable and, during sample extraction or food processing, undergo spontaneous decarboxylation to 6″-O-acetyl-7-O-β-D-glucosides or are hydrolyzed to their 7-O-β-D-glucoside and free isoflavone forms (5, 9). In part because of their instability, the 6″-O-malonyl-7-O-β-D-glucoside and the 6″-O-acetyl-7-O-β-D-glucoside derivatives have limited availability as reference materials and their solutions should be prepared on the day of use.

The primary commercial and dietary sources of isoflavones are foods or preparations containing soy, kudzu, and red clover. Soybeans and soy foods contain primarily daidzein, glycitein, and genistein. Isoflavones are present as aglucones, glucosides, malonyl-7-O-β-D-glucosides, and acetyl-7-O-β-D-glucosides according to the methods used in food processing and preparation of extracts (4, 5, 10, 11).

Red clover is increasingly used as a source of isoflavones in dietary supplement preparations. Formononetin and biochanin A are the most abundant isoflavones in red clover preparations (4, 7, 12), and are present as aglucones, 7-O-β-D-glucosides, and 6″-O-malonyl-7-O-β-D-glucoside derivatives according to the method of processing. Ten different isoflavones (daidzein, genistein, glycitein, formononetin, pseudobaptigenin, calycosin, prunetin, biochanin A, irilone, and pratensein) and a total of 31 derivatives have been identified by LC/tandem mass spectrometry (LC/MS/MS) in an extract of *Trifolium pratense* leaves (13). *P. lobata* (Willd.) Ohwi (common name "kudzu"), a perennial vine native to Japan and China that also grows in the Southeastern portion of the United States, is a commercial source of isoflavones for dietary supplements. The dried root of the herb is used by traditional Chinese physicians to dispel "pathogenic factors" from the superficial muscles, to reduce "heat," to promote salivation, and to relieve thirst (14). It has also been reported to be used internally for colds, influenza, and feverish illnesses (10). The isoflavone content of kudzu is quite complex and seems to have been less studied than those of soy and red clover. Puerarin (daidzein 8-C-β-D-glucoside), daidzin (daidzein 7-O-β-D-glucoside), and daidzein were the primary isoflavones identified in a methanolic extract of *P. radix* (15). Isoflavone diglucosides such as daidzin-4′-O-glucoside and puerarin-4′-O-glucoside, along with other unusual isoflavones, have also been identified in kudzu extracts by LC/MS/MS analysis (6).

Techniques Applied to the Quantitation of Isoflavones

Several techniques have been successfully applied to the analysis of isoflavones (and metabolites) in foods and biological fluids. An extensive review of the techniques applied to phytoestrogen analysis, focusing mainly on the analysis of biological fluids, was published by Wang et al. in 2002 (1). While a qualitative profile of isoflavones can be obtained by direct analysis by matrix-assisted laser desorption-time of flight-MS (MALDI-TOF-MS; 16), a chromatographic separation is necessary before isoflavone quantitation. Several chromatographic techniques may be applied to the separation of isoflavones. Selection of a particular technique will vary depending upon the required sensitivity, chromatographic resolution, and complexity of the matrix under examination. Until recently, gas chromatography (GC) coupled with MS (GC/MS) has been the primary technique applied to isoflavone analysis. LC

coupled with photodiode array (PDA) or MS/MS detection is gaining popularity because it requires less laborious sample preparation than GC and allows the quantitation of unhydrolyzed isoflavone derivatives. Capillary electrophoresis (CE) has also been applied, although less frequently, to the separation of phytoestrogens in extracts of plants such as *P. lobata*.

GC

Isoflavones, as trimethylsilyl (TMS) ether derivatives, can be separated by GC using a nonpolar capillary column and a temperature gradient. A GC procedure for the quantitation of daidzein and genistein extracted from soybeans, as TMS derivatives, was developed by Naim et al. in 1974 (17). Until recently, GC/MS has been the primary technique for the analysis of isoflavones. GC/MS provides high sensitivity, good resolution, and useful information for the identification of unknowns. A disadvantage is that it requires labor-intensive sample preparation. The procedure should include the removal of coextractants that would interfere with the GC separation, and the derivatization of isoflavone free hydroxyl groups into the more volatile and stable TMS ethers. An internal standard should be added in order to account for the significant losses that might occur during this extensive sample preparation. Many methodologies involving GC/MS analysis have used isotopically labeled compounds (^{2}H or ^{13}C) as internal standards (18–20).

HPLC

HPLC utilizing different detectors according to the application needs has gained popularity over the last decade for the analysis of isoflavones. Compared to GC, HPLC requires simpler sample preparation, and hydroxylated compounds do not have to be derivatized prior to analysis. Isoflavones are separated using a reversed-phase column and a gradient of acidified acetonitrile and acidified water. Acetic acid, trifluroacetic acid, and formic acid have all been used for specific applications. Several methods, based on a simple sample preparation followed by HPLC with ultraviolet (UV) detection (LC-UV), were developed for routine analysis of isoflavones. Fluorescence and electrochemical detectors have also been applied, but less frequently than the UV detector over the last decade. Fluorescence detection provides higher sensitivity for some isoflavones, such as daidzein and formononetin, but its application is limited by the nonresponse for isoflavones such as genistein and biochanin A. Post-column derivatization with aluminum of nonfluorescent flavonols and phytoestrogens has been reported, but it was not applicable to all the compounds investigated (21, 22). Aluminum can form a fluorescent complex with flavonols having a free hydroxyl group in position 3 and a keto group in position 4. An electrochemical detector (ED) has also been applied to the analysis of isoflavones as well as other phytoestrogens. Genistein and daidzein were detected with an optimized potential of +0.75 V, while formononetin and biochanin A required a potential of 1.2 V that creates baseline instability due to the electrooxidation of impurities (23, 24). Isoflavones present different voltammograms, according to their structures. An array of EDs can be used for selective measurement of compounds with different reduction or oxidation potentials. Arrays of up to 16 coulometric electrodes have been used for the determination of phenolic and flavonoid compounds in juices and biological samples (25–27). Recently, HPLC-ED has been applied to the determination of isoflavones in soy flour and dietary supplements (11, 28). The acquisition of the entire voltammogram versus the retention time provides additional information for peak identifications and detection of impurities.

HPLC/MS

HPLC/MS has been widely applied to the identification/quantitation of isoflavones, especially in clinical and animal studies. Detailed descriptions and applications of HPLC/MS are reported in recent articles (1, 29). The first application of HPLC/MS to isoflavone analysis was developed by Setchell et al. (24) in 1987 using a thermospray ionization interface. In current methodologies, isoflavones are transferred as ions in the gas phase through the more sensitive and stable atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) interfaces. The analysis of isoflavones by HPLC/ESI-MS in the positive ion mode clearly shows their molecular ions [$M+H^{+}$] (5). More definitive information might be obtained by coupling HPLC with multistage mass spectrometers. With a triple-quadrupole mass detector, the molecular ion of isoflavones is isolated by the first quadrupole, fragmented by collision-induced dissociation (CID), and the daughter fragment ions are then analyzed by the third quadrupole (29). The MS/MS detection can be used for the identification of isoflavones based on their fragmentation pattern, and the application of multiple reaction ion monitoring (MRM) allows highly sensitive quantitative methods of analysis. Griffith and Collison (5) reported that use of an ion trap MS/MS/MS detector with an ESI interface in positive ion mode made possible a nearly unequivocal identification of isoflavones and their derivatives. In the case of 6″-O-malonyl-7-O-β-D-glucosides and 6″-O-acetyl-7-O-β-D-glucosides, the MS/MS spectra provide the aglycone fragment, while the MS/MS/MS spectra provide a recognizable fingerprint that leads to the identification of the isoflavone. While the fragmentation of the isoflavone 7-O-β-D-glucosides leads to the free isoflavone ion, the fragmentation of the 8-C-β-D-glucosides such as puerarin generates a complex pattern without the free isoflavone ion (6). Several studies first identify the compounds of interest by HPLC/MS, and then proceed to the quantitation by HPLC-UV.

CE

CE has been applied less frequently than GC and HPLC to the quantitation of isoflavones. The separation is based on the different mobilities of compounds with electric charges in an electric field along a small diameter capillary (30, 31). Isoflavones are separated based on the fact that, as weak acids,

they achieve negative charge at high pH. Borate buffer is generally applied to the electrophoretic separation because of its interaction with the *cis*-diol moiety of sugars, creating charged complexes. According to Peng and Ye (32), the migration time increases with increasing pH of the running buffer, thereby increasing the resolution of the compounds under investigation (e.g., isoflavones). At the same time, the peak current was found to decrease and peak shapes to deteriorate. The migration time and the resolution were found to increase with increasing buffer concentration but, at the same time, limits of detection increased due to the lower peak current of all analytes and the Joule effect increases. Increasing the applied voltage decreased the migration time but increased the background noise level, which results in a higher limit of detection. Peng and Ye (32) reported that use of a 75 cm × 25 μm id × 360 μm od fused silica capillary with 50 mM borate buffer (pH 9.5) and a voltage of 14 kV provided the optimal parameters for the analysis of isoflavones contained in red clover. Several studies applied capillary zone electrophoresis (CZE) to the quantitation of isoflavones in soy (33, 34) and kudzu (35). CZE has been used for the analysis of isoflavones combined with UV, fluorescence, ED and MS detectors. A more detailed description on the application of this technique to the analysis of phytoestrogens is provided by Wang et al. (1).

Sample Preparation

Extraction

Extraction of isoflavones from foods or dietary supplements is a challenging process. The content of free isoflavone and derivatives can be altered during extraction. Several studies investigated the maximum amount of isoflavones that can be recovered from a specific matrix, by modifying parameters such as extraction solvent, temperature, or pH. Early methods applied methanol (MeOH)–H_2O reflux (36, 37), which was later reported to cause the conversion of the 6″-O-malonyl-7-O-β-D-glucosides and 6″-O-acetyl-7-O-β-D-glucosides to glucoside and aglucone forms. In 1994, Barnes et al. (38) reported that heating, which was used in many previous investigations, is unnecessary and alters the isoflavone composition. According to the authors, the extraction of isoflavones from soy matrixes occurs readily at room temperature using an 80% MeOH solution, and it is complete in 1–2 h (5). Several extraction solvents and conditions were compared by Griffith and Collison (5), who concluded that 60% acetonitrile (MeCN) in deionized (DI) H_2O with a small amount of dimethylsulfoxide (DMSO) at room temperature provides the highest recovery of soy isoflavones. The procedure was simplified by analyzing samples directly after diluting them to 50% MeCN with DI H_2O, while in early studies the extraction solvent was removed and the samples were then reconstituted with 80% MeOH. Umphress et al. (39) recently reported the isoflavone content of 179 soy-containing foods. Food samples were frozen, lyophilized, and ground, and then a portion of 0.2–1.0 g was mixed in a Vortex mixer for 30 min with 30 mL of 80% MeOH. Afterward, the samples were incubated overnight with β-glucosidase before HPLC analysis. In several studies, the extraction was performed by adding concentrated HCl to the extraction solvent. In 1982, Murphy (40, 41), after investigating different extraction solvents, concluded that MeCN with H_2O or with HCl was the most efficient solvent system. However, the reported recoveries of daidzein and coumestrol were less than 63%. The U.S. Department of Agriculture–Iowa State University Isoflavone Database states that "acid addition to extraction solvent" is "highly recommended" (42). Recently developed extraction procedures, aimed at obtaining the least possible alteration of isoflavone derivatives, are conducted at neutral pH and recover the same or higher quantities of isoflavones compared with procedures using acidifying solvents (5). While most of the isoflavone extraction procedures were optimized for soy/soy-containing foods, some modifications might be required when analyzing isoflavones in different matrixes, such as botanical materials. During the extraction of isoflavones from plants, after breaking the cell compartments, the native β-glucosidases are released into the extract and begin to hydrolyze the isoflavone derivatives to their aglucones. In studies targeting the investigation of the isoflavone derivatives in red clover, TRIS (hydroxymethyl) aminomethane buffer was added to the extraction solvent as an inhibitor of the native β-glucosidases (7, 39). During the last several years, the use of automated extraction systems has become more common. Klejdus et al. (43, 44) extracted isoflavones from soy plants and soybeans using 90% MeOH as the solvent at 145°C and 140 bar, repeating the procedure 2 times. As mentioned above, to date, the only method of analysis for isoflavones that has been validated by a collaborative study is AOAC Official Method **2001.10** (3), which is specific for soy-containing foods. In this method, isoflavones are extracted using 80% methanol solvent for 2 h in a shaking thermostatted bath maintained at 65°C.

Internal Standard for Quantitation of Isoflavones by HPLC

The inclusion of an internal standard in the procedure is a critical issue in the development of an analytical procedure for isoflavones, especially when the sample preparation is particularly labor-intensive. Most of the internal standards applied to the analysis of isoflavones from known botanical sources have limited applicability to the analysis of isoflavones from different matrixes. Apigenin (an analog of genistein; 5) and fluorescein (38) have been used in several procedures for the quantitation of isoflavones in soy and soy-containing foods. The application of apigenin was first introduced by Barnes et al. (29) for HPLC/MS analysis. In their method, which was based on an isocratic HPLC separation, apigenin was not resolved from genistein. Apigenin was later separated from genistein by improving the separation conditions through use of an elution gradient, as in the methodology developed by Griffith and Collison (5). Song et al. (45) proposed the synthesis of 2,4,4′-trihydroxydeoxybenzoin (BHT), but the use of this

compound was limited by its lack of commercial availability. With respect to the analysis of isoflavones in red clover, Krenn et al. (12), after evaluating several compounds, selected 6-methoxy-flavanone for their procedure. However, the internal standard was added after the extraction and prior to the HPLC quantitation. Acetophenone, which has also been used as an internal standard (37), might present different extraction selectivity due to its polarity and structural differences from the compounds under investigation. Delmonte et al. (4) proposed the use of 2′-methoxy-flavone and 6-methoxy-flavone as internal standards for the analysis of isoflavones extracted from soy, red clover, and kudzu. Both compounds were proven to be stable under acidic and basic hydrolysis conditions, and did not coelute with any other compound present in the extracts of the investigated samples. The addition of both internal standards in the analysis of samples with unknown composition was suggested in order to check the presence of coeluting compounds by calculating the recoveries ratio of the 2 references. Considering that most of the internal standards used for the analysis of isoflavones show limited applicability to the study of matrixes other than the one under investigation, the absence of any interfering compound(s) in the extract under investigation should be verified before continuing on to an internal standard-based quantitative analysis.

Hydrolysis of Isoflavone Derivatives to Simpler Forms

Almost all of the sample preparations include, prior to HPLC analysis, the hydrolysis of isoflavone derivatives to simpler forms. In certain cases, isoflavones are present as uncharacterized derivatives. In other cases, the reference material is not available, or the chromatographic separation is too complex for direct quantitation. Griffith and Collison (5) developed a methodology for direct quantitation of isoflavones in soy without any hydrolysis step, and included in the method the calibration curves of the acetyl and malonyl derivatives of daidzin, genistin, and glycitin. Their method is limited to the analysis of soy-containing products, and both malonyl and acetyl derivatives were reported to have limited stability in solution. The analysis of preparations containing red clover and kudzu extracts is more complex. Wu et al. (13) identified in the extract of red clover leaves, by HPLC/MS, 31 different isoflavones or derivatives. To date, 15 of them (about 50%) are not available as reference materials.

Figure 2 shows the action of acid/base hydrolysis on conjugated isoflavones. Basic hydrolysis breaks the ester bond of the malonyl and acetyl groups esterified to the alcoholic functions of the sugar moieties, while acidic hydrolysis breaks the carbon-oxygen bond between the isoflavone ring and the sugar.

AOAC Official Method **2001.10** (3), a procedure for the quantitation of isoflavones contained in soy foods, includes a basic hydrolysis step. Soy-containing foods, after basic hydrolysis, are expected to contain only the isoflavones daidzein, glycitein, and genistein, along with their respective 7-β-O-glucosides. Acid hydrolysis, brought about by adjusting the pH of the 80% MeOH extraction solvent to pH 3 with trifluoroacetic acid, was preferred by Krenn et al. (12) for the routine analysis of isoflavones in red clover using HPLC-UV. Delmonte et al. (4) investigated dietary supplements with different compositions, comparing the quantitative results obtained from HPLC-PDA analysis of acid/base hydrolyzed and unhydrolyzed extracts. Higher quantities of total isoflavones were reported in the HPLC-PDA analysis of acid hydrolyzed extracts of samples containing red clover or kudzu. In the same study, it was reported that, under the sample treatment conditions used by the authors, recoveries of the primary isoflavones after acid hydrolysis ranged from 73.4% (genistein) to 101.1% (daidzein). Wang et al. (46) reported previously that the recovery of genistein after acid hydrolysis is consistently affected by the concentration of the HCl used for the hydrolysis and by the reaction time. The recoveries were also affected by the modifications made to the extraction procedure according to the food matrix under investigation. An alternative approach to the acid hydrolysis of isoflavone derivatives is the application of enzymatic hydrolysis (39). Commercial preparations of enzymes, such as β-glycosidases from almonds or *Escherichia coli*, sulfatase and glucuronidase from *Helix pomatia,* were used to hydrolyze isoflavone derivatives into their free forms. However, Ismail and Hayes (47) reported that β–glycosidases do not show the same activity toward all of the isoflavone derivatives. The yield in the free form was particularly low when hydrolyzing 6″-O-malonyl-7-O-β-D-glucosides and 6″-O-acetyl-7-O-β-D-glucosides of soy isoflavones. As a result, enzymatic hydrolysis has a restricted applicability compared to acid hydrolysis, and the enzymatic activity toward all the isoflavone derivatives expected to be present in the extract should be evaluated.

HPLC Quantitation of Isoflavones

Isoflavones are generally separated by reversed-phase HPLC using an RP-18 column and a gradient of MeCN or

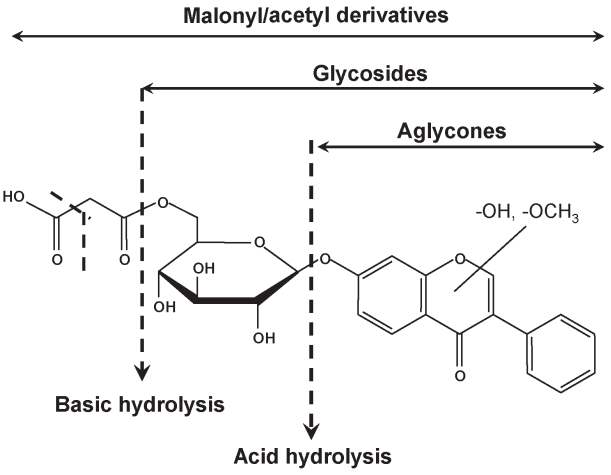

Figure 2. Scheme of acid and base hydrolysis of isoflavone derivatives.

MeOH in H_2O. Both eluents are usually acidified in order to avoid peak-shape degradation caused by the protonation/deprotonation equilibrium of the analyte free hydroxyl groups. Acetic acid, ammonium formate buffers, and formic acid are the most common mobile phase pH modifiers applied to isoflavone analysis. Depending upon the matrix under investigation, and whether or not the sample preparation used includes a hydrolysis step, the separation of isoflavones might be simple or particularly complex. As an example, the separation of the 6 isoflavones present in the base hydrolyzed extract of soy foods, daidzein, glycitein, genistein, and their glucones can be easily achieved on columns having a limited number of theoretical plates. AOAC Official Method **2001.10** (3), a procedure including base hydrolysis and HPLC-UV of isoflavones in soy-containing products, does not specify parameters such as HPLC column brand or size, or elution temperature or flow rate. Other methods, aimed at separation of the isoflavones contained in unhydrolyzed extracts from botanical sources, such as red clover, kudzu, or multi-herbal blends, require a careful optimization of parameters, such as stationary phase chemistry, mobile phase pH and composition, and elution temperature. Wu et al. (13) identified 31 isoflavones in red clover extracts by HPLC/MS, many of them not chromatographically separated. Merken and Beecher (2) summarized all of the chromatographic conditions applied to the separation of isoflavones contained in the extracts from different food matrixes. Figure 3 shows the separation of the primary isoflavones, including their derivatives, found in soy, red clover, and kudzu (with the exception of puerarin) extracts that are available as reference materials. All of the compounds investigated are baseline separated in a 70 min elution. The UV spectra, acquired by a PDA detector, are useful for confirmation of identities and detection of impurities. Slight changes of the elution temperature and eluent pH were found by the authors of this review (data not shown) to quantitatively affect the retention of the 6″-O-malonyl-7-O-β-D-glucoside and 6″-O-acetyl-7-O-β-D-glucoside derivatives. Figure 4 shows the comparison of HPLC separations of the isoflavones extracted from a red clover-based dietary supplement, directly after extraction and after acid or base hydrolysis. The sample preparation and hydrolysis procedures were reported previously (4). The analysis of unhydrolyzed extracts provides more detailed information about the isoflavone profile, but the complex chromatographic separation, along with the instability and limited commercial availability of 6″-O-malonyl-7-O-β-D-glucoside and 6″-O-acetyl-7-O-β-D-glucoside derivatives, makes its application to routine analysis difficult. The analysis of red clover and kudzu dietary supplements after acid hydrolysis showed a higher isoflavone content than when analyzing the unhydrolyzed or base hydrolyzed extracts, possibly because of the inclusion in the quantitation of glycoside derivatives not commercially available for instrument calibration purposes (4). Table 1 shows the quantitation, after base hydrolysis, of isoflavones extracted from dietary supplements of different botanical composition. While the separation of the isoflavone derivatives quantitated in unhydrolyzed extracts is usually performed over a 60–80 min elution, the analysis of hydrolyzed extracts can be performed in a shorter time. Figure 5 shows a faster separation of the primary isoflavones and derivatives quantitated in soy and red clover extracts, divided by chemical structure (and consequently, by reactivity under hydrolysis). Griffith and Collison (5) reported a fast separation of the isoflavones identified in soy/soy foods in less than 12 min using a short chromatographic column at a flow rate of 3 mL/min. The recent developments in the technology of HPLC stationary phases allow improved chromatographic separations in shorter times. To date, the simultaneous HPLC-UV separation of all isoflavones

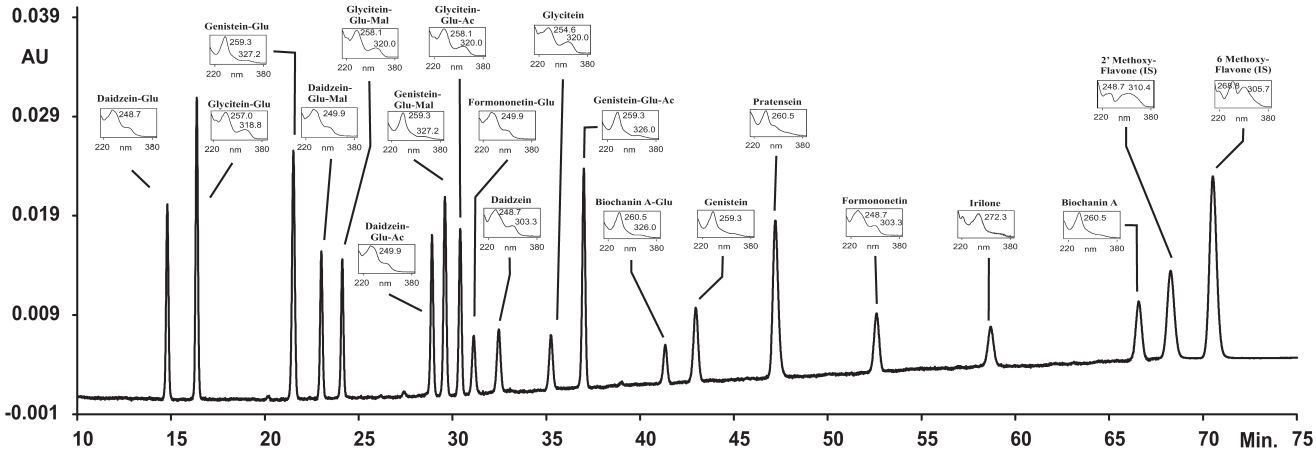

Figure 3. HPLC separation of the primary isoflavones reported in soy, red clover, and kudzu (excluding puerarin) extracts. Conditions as follow: Waters (Milford, MA) Atlantis dC18 column (4.6 × 150 mm, 3 μm) maintained at 45°C; flow rate, 1.0 mL/min; UV detection at 260 nm. Linear elution gradient of 0.1% acetic acid in H_2O (A) and 0.1% acetic acid in MeCN (B), from 9% B to 45% B over 80 min.

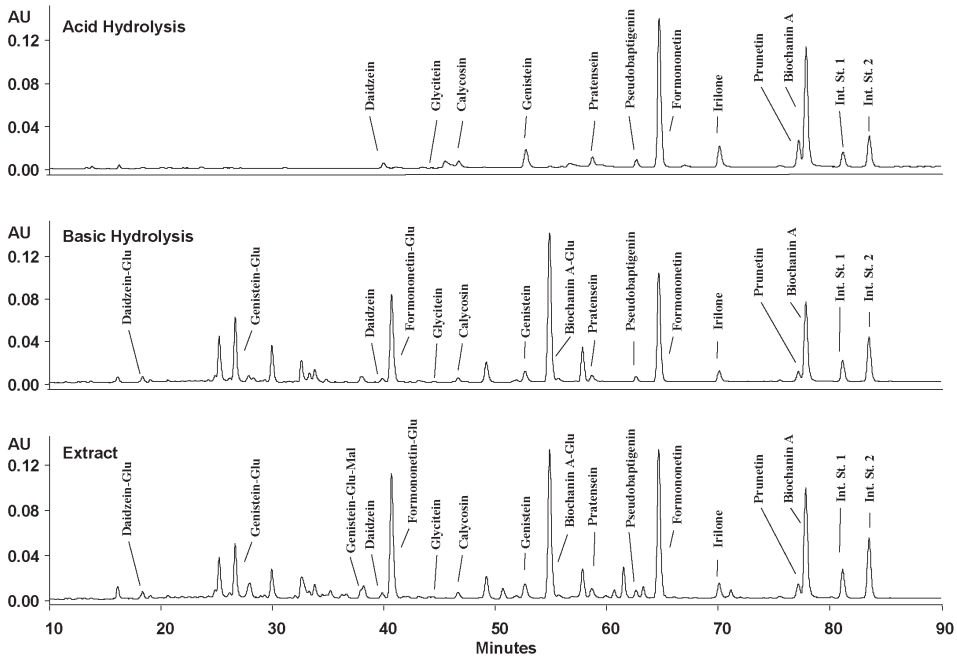

Figure 4. Stacked chromatograms of a red clover-containing dietary supplement. The sample was analyzed directly after extraction (Extract), after base hydrolysis, and after acid hydrolysis. Conditions: a Nova-Pak C18 column (3.9 × 150 mm, 4 μm, Waters) maintained at 40°C, UV detection at 260 nm, flow rate at 1.0 mL/min. Linear elution gradient of 0.1% acetic acid in water (A) and 0.1% acetic acid in acetonitrile (B) as follows: from 5% B to 20% B over 50 min, then from 20% B to 40% B over 40 min. Sample preparation reported in ref. 4.

Table 1. Isoflavone content of selected dietary supplements available in the United States[a]

	Soybean concentrate, soy germ extract, and *radix pueraria* (kudzu) root, mg/g	Fermented soy, dried soybean extract, and red clover flower, mg/g	Red clover, mg/g	Kudzu root, soybeans, and black cohosh extracts, mg/g	Red clover (herb and flower), mg/g	Soy protein concentrate, kudzu extract (root), and red clover extract (leaf), mg/g
Puerarin	65.584	—	—	—	—	49.743
Daidzein	1.613	19.606	0.649	0.311	0.086	2.158
Daidzin	16.230	0.537	0.042	13.095	0.160	8.206
Genistein	0.088	31.444	1.266	0.265	0.175	0.065
Genistin	2.270	1.333	0.171	17.758	1.049	1.245
Glycitein	0.087	—	—	0.234	0.007	0.013
Glycitin	1.372	0.059	—	2.311	0.021	—
Calycosin	—	—	0.897	—	0.127	0.050
Pratensein	0.007	—	1.778	0.010	0.148	0.007
Pseudobaptigenin	0.033	—	1.514	—	0.191	0.046
Prunetin	—	—	2.312	0.043	0.147	0.006
Formononetin	0.070	0.120	24.027	0.064	1.779	0.241
Formnonetin-7-O-β-D-glucoside	0.980	2.644	1.631	0.096	2.438	0.663
Biochanin A	0.004	0.112	40.406	0.119	1.139	0.050
Biochanin A-7-O-β-D-glucoside	0.142	3.753	3.347	0.330	2.783	0.053
Total	88.480	59.608	78.040	34.637	10.250	62.545

[a] Samples were subjected to base hydrolysis before LC-UV quantitation. Preparation of samples as previously reported (ref. 4).

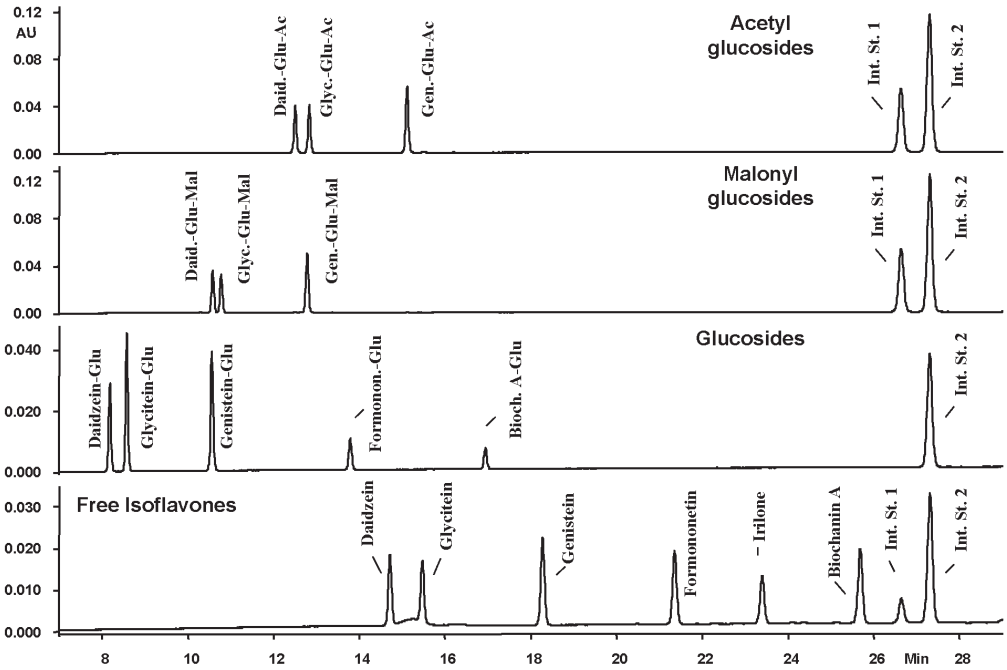

Figure 5. LC fast separation of the primary isoflavones contained in soy, red clover, and kudzu (excluding puerarin) extracts. Chromatographic conditions: Atlantis dC18 column (4.6 × 150 mm, 3 μm, Waters) at 45°C, flow rate 1.0 mL/min, UV detection at 260 nm. Linear elution gradient of 0.1% acetic acid in H_2O (A) and 0.1% acetic acid in acetonitrile (B), from 10% B to 60% B over 30 min.

reported in soy, kudzu, and red clover still requires a long chromatographic elution, or the inclusion of a hydrolysis step in the sample preparation.

Conclusions

Over the last decade, several methodologies for accurate identification and quantitation of isoflavones in food and different botanical sources have been developed. The forms in which isoflavones naturally occur were identified by applying analytical techniques that require minimal sample preparation, such as HPLC/MS and CE. Specifically, the MS/MS and MS/MS/MS spectra obtained by triple quadrupole and ion trap mass detectors permitted the identification of isoflavone derivatives, such as 6″-O-malonyl-7-O-β-D-glucosides and 6″-O-acetyl-7-O-β-D-glucosides, based on their fragmentation patterns. An acid or base hydrolysis step is usually included in the sample preparation in order to transform unstable isoflavone derivatives, such as 6″-O-acetyl- and 6″-O-malonyl-7-O-β-D-glucosides or derivatives for which the reference material is not available, into simpler forms. While the health effects and natural occurrence of the isoflavones found in soy have been intensively studied, little is known about several isoflavones (and derivatives) identified in the extract of plants such as red clover and kudzu that are contained in dietary supplement preparations. To date, only one method for the analysis of isoflavones in foods has been evaluated by a collaborative study, and it is limited to the quantitation of the isoflavones found in soy.

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