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Analysis of Soy Isoflavones in Foods and Biological Fluids: An Overview

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ABSTRACT

Soy isoflavones are phytoestrogens mainly present in soybean and soybean products. Isoflavones have been reported to possess physiological activities such as inhibition of cancer cell proliferation, reduction of cardiovascular risk, prevention of osteoporosis and alleviation of postmenopausal syndrome. This paper gives an overview of soy isoflavone analysis in foods and biological fluids. Extraction of isoflavones is often carried out by polar solvents such as methanol, ethanol, acetone or acetonitrile, or in combination with acid-containing water. The separation, identification and quantification of isoflavones are usually conducted by gas chromatography coupled with flame ionization detector or mass spectrometer (GC/FID or GC/MS), high-performance liquid chromatography with UV or mass spectrometer (HPLC/UV or HPLC/MS) and immunoassay. GC methods provide high sensitivity and adequate separation of only few soy isoflavones; however, a time-consuming derivatization step is needed before analysis. HPLC with a gradient solvent system with or without acid has been applied to analyze soy isoflavones, but most HPLC methods failed to separate all 12 isoflavones in a single run or the separation time is lengthy. Nevertheless, an appropriate choice of mobile phase and gradient condition in a C18 column with UV detection could provide a simultaneous separation of 12 soy isoflavones within short time. For immunoassay, it is convenient, fast, highly sensitive and can analyze a large number of samples at the same time. Yet, the drawback is that only a few soy isoflavones can be determined.

Key words: isoflavones, GC/MS, HPLC/MS, immunoassay

INTRODUCTION

Isoflavones are phytoestrogens that exist mainly in soybean and its products. The structure of soy isoflavones resembles 17 β-oestradiol (a female hormone) and has been reported to possess many biological functions like reduction of menopausal symptoms[(1)]. One of the proposed mechanisms was attributed to its interaction with the estrogen receptors, ERα and ERβ, identified in human[(2,3)]. It has been well documented that isoflavones could inhibit cancer cell proliferation[(4,5)], enhance antioxidant and anti-inflammation activities[(6-8)], prevent osteoporosis[(9,10)] as well as coronary heart disease[(11)]. Also, the intake of soy isoflavones may be closely associated with prevention of hormone-dependent cancers, prostate cancer and breast cancer. For instance, Japanese people consuming soy-rich foods were found to have a lower incidence and mortality of breast cancer[(12)]. The occurrence of mammary tumors was lower for rats consuming a soy-based diet than that without soy[(13)]. The inhibition mechanism of human breast cancer cell lines by genistein, one of the major isoflavones in soy, could be due to its anti-angiogenesis effect[(14,15)]. Among all soy isoflavones, the aglycones, namely, 4',7-dihydroxyisoflavones (daidzein), 4',5,7-trihydroxyisoflavones (genistein) and 4',7-dihydroxy-6-methoxyisoflavone (glycitein) are discussed most as they were reported to exhibit higher biological function than the other isoflavones[(16,17)]. Both genistein and daidzein have received great interest as dietary phytoestrogens that may be used for menopausal hormone replacement therapy[(18,19)].

The concentration of isoflavones in soybean is about 0.2-0.4% (w/w), which is influenced by variety, place of origin, growth environment and harvest season. Isoflavones in soybean can be divided into 4 chemical forms: aglycone (daidzein, genistein and glycitein), glucoside (daidzin, genistin and glycitin), acetylglucoside (acetyldaidzin, acetylgenistin and acetylglycitin), and

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malonylglucoside (malonyldaidzin, malonylgenistin and malonylglycitin) (Figure 1). Approximately 97-98% of isoflavones in plants are naturally present in glycosidic form and can be hydrolyzed by glycosidase or acid or base to produce their aglycones[20].

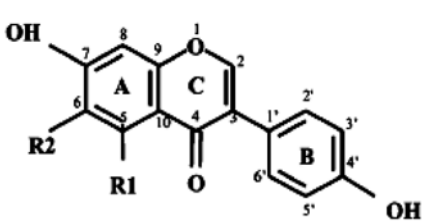
In view of the impact of isoflavones to human health, the development of an appropriate extraction, purification, separation, identification and quantitation method of isoflavones in food products and biological fluids is extremely important. Solvents used for isoflavone extraction often include acetonitrile, acetone, methanol or ethanol, which are used alone or in combination with water or acid[21,22]. The separation, identification and quantification of soy isoflavones are often conducted by high-performance liquid chromatography (HPLC) or gas chromatography (GC). The former method is often coupled with UV, photodiode-array (DAD) or mass spectrometric (MS) detectors, while the latter coupled with flame ionization detector (FID) or MS detector. In addition, the immunoassay is adopted to analyze isoflavones in food products and biological samples. Nevertheless, each method has advantages and limitations. The objective of this paper is to provide an overview of isoflavones analysis in food products and biological fluids.

HPLC ANALYSIS FOR SOY ISOFLAVONES

I. *Extraction*

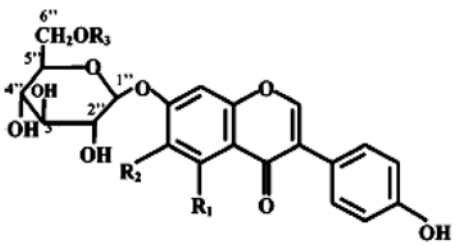
The extraction of soy isoflavones from foods is often carried out by polar solvents such as ethanol, methanol or acetonitrile or in combination with water or acid. However, most methods required a time-consuming clean-up procedure to minimize the interference during subsequent analysis. Griffith and Collison[23] extracted 6 isoflavones (daidzin, glycitin, genistin, daidzein, glycitein and genistein) from soy-containing foods and nutritional supplements by mixing 1 g of sample with 10 mL of acetonitrile and 6 mL of deionized water, followed by shaking for 2 h, centrifuging at 2000 ×g for 10 min and filtering through a 0.45 μm PVDF filter for HPLC analysis. A high recovery of 6 isoflavones ranging from 99 to 101% was attained.

For soy nutrition foods, Chen *et al.*[24] compared extraction efficiency of different solvents including acetonitrile, ethanol and methanol, with the highest recovery (92.4-111.0%) being shown for acetonitrile by sonication at 25°C for 40 min, followed by ethanol and methanol. In addition, with acetonitrile as solvent, the optimum sonication time and refluxing time were 20-50 min and 60 min, respectively. However, with prolonged treatment, the hydrolysis of acetyl isoflavones may occur. Rostagno *et al.*[25] extracted 12 isoflavones from foods by sonication with 50% ethanol at 60°C, followed by purification using a SPE cartridge with 100% methanol as eluent, and a mean recovery ranging from 98.7-100.7% was obtained. Klejdus *et al.*[26] evaluated several methods, including sonication (SN; 38 kHz, 150 W), soxhlet (ST; 130°C for 1 h) and a combination of sonication and pressurized liquid extraction (SN-PLE) for isoflavone extraction from soy bits with 90% aqueous methanol as solvent. The SN-PLE procedure involves sonication first followed by PLE under N_2 with temperature at 140°C and pressure at 140 bar. For daidzin, glycitin, genistin, daidzein, glycitein and genistein, the SN-PLE method yielded a higher recovery (97-104%) than SN (10.9-28.9%) and ST (57.7-119.8%).



Aglycones

R_1	R_2	Compound
H	H	daidzein
OH	H	genistein
H	OCH_3	glycitein



Glucosides

R_1	R_2	R_3	Compound
H	H	H	daidzin
OH	H	H	genistin
H	OCH_3	H	glycitin
H	H	$COCH_3$	6"-O-acetyldaidzin
OH	H	$COCH_3$	6"-O-acetylgenistin
H	OCH_3	$COCH_3$	6"-O-acetylglycitin
H	H	$COCH_2COOH$	6"-O-malonyldaidzin
OH	H	$COCH_2COOH$	6"-O-malonylgenistin
H	OCH_3	$COCH_2COOH$	6"-O-malonylglycitin

Figure 1. Chemical structures of isoflavones.

Four different extraction solvents for soy isoflavones were compared by Penalvo *et al*.[27]: (1) 80% ethanol at room temperature, (2) 80% ethanol at different temperatures (60-100°C) and incubation times (0.5-4 h), (3) 80% methanol followed by saponification (0.15 M NaOH), (4) 80% ethanol in 1 M HCl followed by incubation at 80°C for 1 h. Treatments 1 and 2 showed no significant hydrolysis of acetylglucoside and malonylglucoside at 60°C, but with temperature raised to 100°C, a complete loss occurred. The saponification step in treatment 3 caused complete hydrolysis of acetylglucoside, while both acetylglucoside and malonylglucoside were hydrolyzed in treatment 4. However, the treatment 4 was claimed to the most appropriate method because of both high yield and recovery. Likewise, Kao *et al*.[22] evaluated several polar solvents without or with 0.1 M HCl and concluded that the recovery of most isoflavones was higher for solvents in combination with acid, with a maximum recovery being achieved with acetone-0.1 M HCl (66.3-85.5%) and methanol-ethyl acetate-acetone (1: 1: 1, v/v/v)-0.1 M HCl (75.5-81.1%). Similar result was shown for extraction of daidzein and genistein from tofu, soy milk and processed soybean by using 96% ethanol in 2 M HCl[28]. From the results shown above, the application of acid-containing solvent and high-temperature treatment during extraction can greatly enhance both recovery and yield of isoflavones, however, both may also promote hydrolysis of acetyl and malonyl forms of isoflavones, altering the composition of isoflavones in foods.

Despite the high recovery that can be acquired by solvent extraction, the use of organic solvents may be harmful to environment. Owing to its safety and environment-friendly nature, supercritical carbon dioxide is another solvent that can be used for soy isoflavone extraction. But, the non-polar characteristic of carbon dioxide can decrease extraction efficiency. To remedy this problem, several modifiers like ethanol and water are incorporated to enhance polarity of supercritical carbon dioxide during extraction to elevate the extraction efficiency[21,25,29]. In a recent study, Kao *et al*.[21] compared solvent extraction (SE) and supercritical fluid extraction (SFE) of isoflavones from soybean cake. Compared to SFE at 80°C and 350 bar with ethanol-water (70: 30, v/v) as modifier, the SE method with water-ethanol (1: 1, v/v) as solvent showed a higher yield of total isoflavone, with the levels of total glucoside and malonylglucoside being higher for SE, and total aglycone and acetylglucoside for SFE. This phenomenon clearly indicated that the non-polar nature of carbon dioxide impedes the extraction of polar isoflavones by SFE, which could be overcome by adding an appropriate modifier like water and/or ethanol.

As the isoflavone content in human body fluids can be used as a biomarker in epidemiological studies, developing a rapid and sensitive analytical method for determination of soy isoflavones in human plasma and urine is essential. Both Grace *et al*.[30] and Chan *et al*.[31] employed a SPE cartridge to extract and purify isoflavones from human urine and serum, respectively. Urine or serum sample was initially mixed with 216 μL of 0.14 M sodium acetate buffer solution (pH 5.0), followed by adding 24 μL of β-glucuronidase/aryl sulphatase for hydrolysis and incubating at 37°C. The hydrolyzed sample was then subjected to SPE using a C18 cartridge. The elution solvent, ethyl acetate-acetonitrile (1: 1), was used for urine sample, while a mixture of 0.1% formic acid in methyl tert-butyl ether (MTBE) and methanol (80: 20, v/v) for the serum sample, with the recoveries of daidzein, genistein and glycitein being 100.7, 100.0 and 104.3% for the former and 100.0, 92.0 and 101.0% for the latter. Similarly, Chen *et al*.[32] used MTBE as extraction solvent for urine sample and the average recoveries of genistein, daidzein and glycitein were 110.4, 103.7 and 92.9%, respectively, for a spiked concentration at 100 mg/mL, but declined to 98.2, 82.3 and 95.7% for 2 μg/mL.

II. *Separation Methods*

Table 1 summarizes various HPLC methods along with the conditions reported for determination of soy isoflavones in both foods and body fluids. Hutabarat *et al*.[28] resolved 2 soy isoflavones (daidzein and genistein) along with formononetin, biochanin A and coumestrol from soybean using a Phenyl Nova-Pak column (150 mm × 3.9 mm i.d., 4 μm particle size) and an isocratic mobile phase of acetonitrile-water (33: 67, v/v) at a flow rate of 1 mL/min within 24 min. This method is simple and less detrimental to column, but only two soy isoflavones were determined. Zheng and Row[33] developed a RP-HPLC method for separation of 8 isoflavones (daidzin, glycitin, genistin, 6”-O-acetyldaidzin, 6”-O-malonylgenistin, daidzein, genistein and glycitein) by employing a C18 column (250 mm × 4.6 mm i.d., 5 μm particle size) and a mobile phase of (A) 0.1% acetic acid in water and (B) 0.1% acetic acid in acetonitrile with the following gradient condition: 88% A and 12% B in the beginning and maintained for 9 min, increased to 15% B in 19 min, 27% B in 30 min and 35% B in 50 min. The flow rate was 1.0 mL/min with UV detection at 254 nm. A total of 8 soy isoflavones were separated, but the method is relatively lengthy.

In another study, Qu *et al*.[34] separated 6 soy isoflavones (daidzin, glycitin, genistin, daidzein, genistein and glycitein) from *semen*, a popular Chinese herb product produced by fermentation of soybean. A Lichrospher C18 column (150 mm × 6.0 mm i.d., 5 μm particle size) and an isocratic solvent system of methanol-water-acetic acid in two different ratios, 30: 70: 2 and 45: 55: 2 (v/v/v), was employed for separation of glycosides and aglycones, respectively, with flow rate at 1.0 mL/min and UV detection at 260 nm. However, this method failed to separate 6 soy isoflavones in a single run and the number of compounds separated is less.

A fast HPLC method with UV detection was

Table 1. HPLC methods for determination of soy isoflavones in body fluids and foods

Extraction	HPLC column	Separation condition	References
Sample extracted with 80% ethanol and then applied into a SPE cartridge for elution with 80% methanol, dried, redissolved in mobile solvents.	Waters BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm)	A: 0.3% acetic acid in water B: methanol; flow rate: 0.8 mL/min; UV at 270 nm	Klejdus *et al*. (2008)
Isoflavone standards	RS-tech C18 column (46 mm × 25 mm i.d., 5 μm) from Daejeon, Korea	A: 0.1% acetic acid in water B: 0.1% acetic acid in acetonitrile; flow rate: 1 mL/min; UV at 254 nm	Zheng and Row (2007)
Sample extracted by supercritical fluid extraction, followed by refluxing with 70% ethanol, drying, redissolving in water and then chromatographed on a D101 macroporous resin by eluting with water, 40%, 75% and 95% ethanol.	Lichrospher C18 column (150 mm × 6.0 mm i.d., 5 μm) from Hanban Science, China	Methanol-water-acetic acid (30: 70: 2, v/v/v) for glycoside fraction and 45: 55: 2 (v/v/v) for aglycone fraction; flow rate: 1 mL/min; UV at 260 nm	Qu *et al*. (2007)
Sample was sonicated with 50% ethanol in water and centrifuged.	Chromolith monolithic RP-18e column from Merck	A: 0.1% acetic acid in water, B: 0.1% acetic acid in methanol; flow rate: 5 mL/min; UV at 254 nm	Rostagno *et al*. (2007)
Urine was extracted with methyl *tert*-butyl ether, centrifuged, dried, redissolved in methanol-0.05 mol/L ammonium formate (50: 50, v/v).	Luna C18 column (150 × 4.6 mm i.d., 5 μm) from Phenomenex	A: 0.05 mol/L ammonium formate (pH 4) B: acetonitrile; SIM/MS	Chen *et al*. (2007)
Serum was hydrolyzed first, followed by extracting in a SPE cartridge with 0.1% formic acid in methyl *tert*-butyl ether and methanol (80/20, v/v) as elution solvent, drying and redissolving in methanol-water (50/50, v/v).	Agilent Zorbax Bonus-RP C18 column (150 mm × 2.1 mm i.d., 5 μm)	A: 0.3% acetic acid in water B: 0.3% acetic acid in acetonitrile; flow rate: 0.2 mL/min; SIM/MS	Chan *et al*. (2006)
Sample was filtered and sprinkled with SPE-ed^{TM} matrix for extraction under two-step controlled conditions: (1) preheated and extracted twice with hexane, followed by subjecting to pressurized nitrogen; (2) preheated and extracted twice with 90% methanol, followed by subjecting to pressurized nitrogen. The extracts were centrifuged, dried, redissolved in methanol and filtered.	Atlantis C18 column (20 mm × 2.1 mm i.d., 3 μm)	A: 0.1% acetic acid in water (pH 3.75) B: methanol; flow rate: 0.35 mL/min; DAD at 254 nm	Klejdus *et al*. (2005)
Sample extracted with 80% methanol by sonication and filtered.	Phenomenex Prodigy ODS column (150 × 3.2 mm i.d., 5 μm) from Phenomenex Inc., Torrace	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile; flow rate: 1.0 mL/min; UV at 254 nm and ESI/MS	Wu *et al*. (2003)
Soybean powder extracted with acetone in 0.1 M hydrochloric acid (5: 1, v/v), shaken (2 h), centrifuged, supernatant dried, redissolved in methanol and filtered.	Vydac 201TP54 C18 column (250 × 4.6 mm, i.d., 5 μm)	A: water and B: acetonitrile; flow rate: 2.0 mL/min; UV at 262 nm	Kao and Chen (2002) Hsieh *et al*. (2004)
Diluted serum/plasma poured into the SPE cartridge for elution with acetonitrile, dried, redissolved in methanol.	Ultracarb ODS column (150 mm × 2 mm i.d., 3 μm) from Phenomenex, Torrance, CA	A: 0.1% formic acid in water B: acetonitrile; flow rate: 0.2 mL/min; ESI/MS/MS	Twaddle *et al*. (2002)
Sample extracted with 80% methanol (pH 3 with TFA), filtered, dried, redissolved in dimethylsulfoxide.	Hypersil BDS-C18 column (250 × 4 mm i.d., 5 μm) from Shandon, Runcorn, UK	A: water (adjusted to pH 2.7 with sulfuric acid) B: acetonitrile; flow rate: 1 mL/min; UV at 254 nm.	Krenn *et al*. (2002)

Table 1. Continued.

Extraction	HPLC column	Separation condition	References
Urine sample poured into a SPE C18 cartridge for elution with water-methanol (85: 15, v/v) and subjected to solid-phase microextraction (SPME).	Waters Symmetry C18 column (50 × 2.1 mm i.d., 3.5 μm)	A: methanol and B: water; flow rate: 0.1 mL/min; ESI/MS	Satterfield *et al.* (2001)
Plasma or urine hydrolyzed and extracted with methyl *tert*-buty ether.	Luna Phenyl-Hexyl column (150 × 4.6 mm i.d., 5 μm)	A: 0.05 M ammonium formate B: methanol/acetonitrile (50: 50, v/v)	Thomas *et al.* (2001)
Sample extracted with acetonitrile and deionized water, followed by diluting with water to make the final acetonitrile concentration to 50% (v/v), centrifuging and filtering.	Standard method: YMC ODS-AM (150 × 3 mm i.d., 3 μm) Rapid method: Alltima C18 Rocket column (53 × 7 mm i.d., 3 μm)	A: 0.1% acetic acid in water B: 0.1% acetic acid in acetonitrile; flow rate: 0.65 mL/min; UV at 260 nm.	Griffith *et al.* (2001)
Sample extracted with 2 M HCl and 96% ethanol, centrifuged and filtered.	Phenyl Nova-Pak column (150 × 3.9 mm i.d., 4 μm)	A: acetonitrile and B: water; flow rate: 1 mL/min; DAD (200-400 nm)	Hutabarat *et al.* (2000)
Sample extracted with 80% aqueous methanol by sonication and stirring, followed by centrifuging and diluting (1: 1) with 0.2 M acetate buffer.	Waters NovaPak C18 column (150 × 3.9 mm i.d., 4 μm)	A: acetic acid-water (10: 90, v/v) B: methanol-acetonitrile-dichloromethane (10: 5: 1, v/v/v); flow rate: 0.8 mL/min; DAD at 260 and 280 nm	Franke *et al.* (1999)
Samples extracted twice with diethyl ether, dried, redissolved in 25% methanol containing 10 mM ammonium acetate and 71 mM triethylamine.	Supelco Discovery RPamide-C16 column (25 × 4.6 mm i.d., 5 μm)	A: 25% methanol containing 10 mM ammonium acetate and 71 mM triethylamine (pH 4.5) B: 95% methanol containing 10 mM ammonium acetate and 71 mM triethylamine (pH 4.5); flow rate at 1.0 mL/min; MS	Cimino *et al.* (1999)

developed by Klejdus *et al.*[26], with 6 soy isoflavones resolved from soy bits within 8 min by employing an Atlantis C18 column (20 mm × 2.1 mm i.d., 3 μm particle size) and a mobile phase of (A) 0.1% acetic acid (pH 3.75) and (B) methanol at a flow rate of 0.35 mL/min, with column temperature at 36°C. The gradient condition used was: 87% A and 13% B initially, rose to 22% B in 2.5 min, 30% B in 3.21 min, 35% B in 4 min, 40% B in 4.5 min, 50% B in 5.14 min and then decreased to 13% B in 7.71 min. The detection limits of daidzein, genistein, glycitein, daidzin, genistin and glycitin were 1.6, 1.1, 2.0, 3.0, 1.5 and 2.9 ng/mL, respectively. Though this method is relatively fast (8 min), only 6 isoflavones were separated and the peaks for daidzein and glycitein as well as daidzin and glycitin are not adequately resolved.

In a later study, an ultra-high pressure liquid chromatography (UPLC) method was developed by Klejdus *et al.*[35] to analyze both soy isoflavones and phenolic acids. A total of 6 soy isoflavones were separated within 1.9 min by employing a Waters BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm particle size) and a mobile phase of (A) 0.3% acetic acid in water and (B) methanol with the following gradient condition: 78% A and 22% B initially, raised to 50% B in 1.0 min, 100% B in 1.4 min and returned to 22% B in 1.8 min. The flow rate was 0.8 mL/min, with UV detection at 270 nm and column temperature at 60°C. The detection limits of daidzin, glycitin, genistin, daidzein, glycitein and genistein were 0.256, 0.223, 0.189, 0.237, 0.304 and 0.284 ng/mL, respectively. This method is fast, but it analyzed only 6 soy isoflavones and did not adequately resolve daidzin and glycitin.

All the 12 soy isoflavones were successfully separated from soy-based foods by Franke *et al.*[36], employing a Waters NovaPak C18 column (150 mm × 3.9 mm i.d., 4 μm particle size) and a mobile phase of (A) acetic acid-water (10: 90, v/v) and (B) methanol-acetonitrile-dichloromethane (10: 5: 1, v/v/v) with flow rate at 0.8 mL/min, UV

detection at both 260 and 280 nm. The gradient elution was 95% A and 5% B initially for 5 min, rose to 45% B in 25 min, 70% B in 31 min and dropped to 5% B in 34 min. For several soy foods commonly consumed in Singapore and Hawaii, the mean total isoflavone content ranged from 35 to 7500 μg/mL, with the level being highest in soybean and soybean seeds. The total isoflavone content and conjugation patterns are varied depending on soybean variety, environmental condition, storage and processing condition. This method allows adequate separation of 12 soy isoflavones, the mobile phase is complex and the separation time is still long.

In another study, Penalvo *et al*.[27] developed a relatively simple HPLC method with coulometric electrode array detection to analyze isoflavones from soy products. An Inertsil ODS-3 column (150 mm × 3 mm i.d.) coupled with a C18 guard column (10 mm × 3 mm i.d., 5 μm particle size) and a mobile phase consisting of (A) 50 mM sodium acetate buffer (pH 5)/methanol (80: 20, v/v) and (B) 50 mM sodium acetate buffer (pH 5)-methanol-acetonitrile (40: 20: 20, v/v/v) was used with the following gradient condition: 70% A and 30% B initially, maintained for 15 min, raised to 80% B in 35 min, 100% B in 40 min, maintained till 45 min, returned to 30% B in 47 min and maintained until 65 min. All 12 soy isoflavones were separated within 52 min and the detection limits of 6 soy isoflavones, including glucosides and aglycones, ranged from 0.40 to 1.02 ng/mL. The levels of total isoflavones in three different soy samples were from 37.9 to 446.0 mg/g. The advantage of this method is that all the 12 soy isoflavones are resolved simultaneously, but it takes long separation time and complicated mobile phase.

For determination of soy isoflavones in soy-containing foods and nutritional supplements, Griffith *et al*.[23] developed two different HPLC methods: the first method (standard) employed a YMC ODS-AM column (250 mm × 3 mm i.d., 5 μm particle size) and a mobile phase of (A) 0.1% acetic acid in water and (B) 0.1% acetic acid in acetonitrile, with a linear gradient elution of 90% A and 10% B initially, increased to 30% B in 60 min. The flow rate was 0.65 mL/min with detection at 260 nm. The second method (rapid) used an Alltima C18 Rocket column (53 mm × 7 mm i.d., 3 μm particle size) and a solvent system of (A) 0.1% acetic acid in water (adjusted to pH 3.5 with ammonium hydroxide) and (B) acetonitrile with a linear gradient condition: 73% A and 27% B initially for 9 min, raised to 29% B in 13 min, 90% B in 15 min, and equilibrated for 2 min at 10% B between runs. The flow rate was 3 mL/min with detection at 260 nm. Compared to the second method, the standard method provided better resolution, but the separation time (54 min) was long. Even though the rapid method could overcome this limitation, it failed to provide adequate resolution for isoflavones in food system because of matrix complexity.

By employing a Vydac 201TP54 C18 column (250 mm × 4.6 mm i.d.) and a mobile phase of (A) acetonitrile and (B) water, Kao and Chen[22] resolved 12 soy isoflavones within 20 min. The gradient condition used was: 5% A and 95% B initially, increased to 10% A in 5 min, 25% A in 15 min, 32% A in 27 min, with a flow rate at 2.0 mL/min, column temperature at 25°C and UV detection at 262 nm. The detection limits of daidzein, glycitein, genistein, daidzin, glycitin, genistin, acetyldaidzein, acetylglycitein and acetylgenistein ranged from 0.07 to 0.14 μg/g. To further shorten the separation time, Hsieh *et al*.[37] modified the column temperature as 35°C and gradient elution as follows: 8% A and 92% B initially, increased to 10% A in 2 min, 12% A in 3 min, 22% A in 10 min, 23% A in 11 min, 35% A in 12 min, 50% A in 13 min, maintained for 3 min, and returned to 8% A in 20 min. All the 12 isoflavones were separated within 15 min, with a substantial improvement in detection limits (ranged from 0.03 to 0.11 μg/g), which were lower than that reported by Kao and Chen[22].

In a recent study conducted by Rostagno *et al*.[38], the separation time for 12 soy isoflavones was further reduced to within 10 min by a monolithic column (Chromolith RP-18e from Merck) and a mobile phase of (A) 0.1% acetic acid in water and (B) 0.1% acetic acid in methanol with the following gradient condition: 100% A and 0% B initially, decreased to 69% A in 2 min and maintained until 4 min, 65% A in 5 min and maintained until 8 min, and dropped to 0% A in 9.5 min. The column temperature was 35°C, with a flow rate at 5 mL/min and UV detection at 254 nm. This method can determine the levels of individual soy isoflavones in soy-based foods. Nonetheless, the mobile phase used is complex and the high flow rate at 5 mL/min may reduce column life. For determination of soy isoflavones in urine sample, Cimino *et al*.[39] developed an HPLC/MS method using a Supelco Discovery RPamide-C16 column (25 mm × 4.6 mm i.d., 5 μm particle size) and a mobile phase of (A) 25% methanol containing 10 mM ammonium acetate and 71 mM triethylamine (TEA) (pH 4.5) and (B) 95% methanol containing 10 mM ammonium acetate and 71 mM TEA (pH 4.5). The gradient elution used was 35% A and 65% B initially, increased to 95% B in 10 min, maintained for 1 min at a flow rate of 1.0 mL/min, with total run time within 11 min. About 24 h after ingestion of soy-diet, the levels of genistein and daidzein were 10,259 and 12,303 nmol/L in rat urine, and 21,167 and 24,776 nmol/L in human urine, respectively, with detection limit of 5 ng/mL for both isoflavones. This method is rapid and only needs a small volume of urine sample (0.5-1 mL), but only several soy isoflavones are analyzed and the resolution is inadequate. A picogram level of isoflavones detection could be achieved using an HPLC-ESI-MS method developed by Satterfield *et al*.[40]. A Waters Symmetry C18 column (50 mm × 2.1 mm i.d., 3.5 μm particle size) and an isocratic mobile phase of methanol-water (55: 45, v/v) with a flow rate at 0.1 mL/min were used at room temperature, with the total separation time

being within 10 min. The detection limits of daidzein and genistein were 25.4 and 2.7 pg/mL, respectively, and both levels in urine of volunteers were the same at 6 ng/mL when fed daily with 35 g of soy protein. This method provides a high sensitivity, yet only aglycone isoflavones (daidzein and genistein) are separated.

Thomas *et al*.[41] developed two different methods for determination of soy isoflavones in human plasma and urine. For plasma analysis, a Luna Phenyl-Hexyl column (150 mm × 4.6 mm i.d., 5 μm particle size) and a mobile phase of (A) 0.05 M ammonium formate (pH 4.0) and (B) methanol-acetonitrile (50: 50, v/v) was used with the following gradient elution: 100% A and 0% B initially, increased to 40% B in 0.5 min and maintained until 11.5 min, 80% B in 12.5 min and maintained until 15.5 min. The flow rate was 2 mL/min with detection at 259 nm. For urine analysis, a Zorbax Eclipse XDB-Phenyl HPLC column (75 mm × 4.6 mm i.d., 3.5 μm particle size) and a mobile phase of (A) 0.05 M ammonium formate (pH 4.0) and (B) methanol was used with the following gradient condition: 90% A and 10% B initially for 0.5 min, increased to 45% B in 1 min and maintained until 10.5 min, 80% B in 11.5 min and maintained until 14.5 min. The flow rate was 2 mL/min with detection at 259 nm. The quantitation limits of daidzein, genistein and glycitein were approximately 2 ng/mL. The sensitivity of this method is high; however, only aglycones (daidzein, genistein and glycitein) are analyzed.

Twaddle *et al*.[42] developed a high throughput LC-ES/MS/MS (electrospray tandem mass spectrometry) method for determination of soy isoflavones in human and rodent blood. An Ultracarb ODS column (150 mm × 2 mm i.d., 3 μm particle size, Phenomenex, Torrance, CA) and an isocratic solvent system of 0.1% formic acid-acetonitrile (65: 35, v/v) were employed at a flow rate of 0.2 mL/min with total run time within 10 min. The quantitation limit of both genistein and daidzein was 0.005 μM. Serum samples from male volunteers were analyzed after a daily ingestion of commercial soy diet for 3 months, with the levels of genistein, daidzein and equol being 0.280, 0.160 and 0.045 μM, respectively. This method is rapid and the mobile phase is simple, but only a small number of isoflavones are separated.

Chan *et al*.[31] quantified 3 soy isoflavone aglycones in human serum by an HPLC/ESI/MS method with an Agilent Zorbax Bonus-RP C18 column (150 mm × 2.1 mm i.d., 5 μm particle size) and a mobile phase of (A) 0.3% acetic acid in water and (B) 0.3% acetic acid in acetonitrile at a flow rate of 0.2 mL/min with the following gradient elution: 65% A and 35% B initially, raised to 75% B in 20 min, 90% B in 25 min, lowered to 35% B in 30 min and maintained until 40 min. The detection limits of glycitein, daidzein and genistein were 0.27, 0.38 and 0.29 ng/mL, respectively, and their levels in serum samples from postmenopausal women fed with a single dose of 40 g of soy foods were 39.4, 356.7 and 383.8 ng/mL. This method allows determination of aglycones (glycitein, daidzein and genistein) in human serum; however, the separation time is long and only 3 isoflavones are analyzed.

In a recent study, an improved HPLC/APCI/MS method was developed by Chen *et al*.[32] to analyze soy isoflavones and their metabolites in human urine by a reversed-phase Luna C18 column (150 mm × 4.6 mm i.d., 5 μm particle size) and an isocratic mobile phase of (A) 0.05 mol/L ammonium formate (pH 4) and (B) acetonitrile, with total run time within 40 min. The detection limits of dihydrodaidzein, 3'-hydroxydaidzein, glycitein, daidzein, genistein, dihydrogenistein, and *O*-desmethylangolensin were 37.0, 23.5, 12.2, 15.4, 14.8, 2.2 and 0.3 pmol/L, respectively. This method provides high sensitivity and specificity for isoflavone analysis, but the number of compounds separated is less.

PARAMETERS AFFECTING THE SEPARATION EFFICIENCY BY HPLC

I. *Stationary Phase of the Column*

Generally, a non-polar reversed-phase C18 column is used with a polar mobile phase containing a mixture of water and acetonitrile or methanol. Conversely, with a polar normal-phase column containing silica gel as stationary phase, a poor resolution may occur as a result of strong hydrophilic interaction between isoflavone and silica gel.

II. *Particle Size of Column Packing Material*

Packing materials of particle sizes between 3 and 5 μm are frequently used with columns of varied lengths for separation of isoflavones. Theoretically, the smaller the particle size and the longer the column, the better is the resolution of isoflavones. However, a 15-cm long column with 3-μm particle would provide a comparable separation efficiency of a 25-cm long column with 5-μm particle, as the theoretical plates for both are similar[23,27].

III. *Column Diameter*

Just like the particle size, the smaller the column diameter, the better is the separation efficiency. However, the sample size has to be reduced substantially for a narrow column. Based on the literature reports discussed above, a 15 or 25-cm long column with a particle diameter ranging from 3 to 4.6 mm is most often used for separation of isoflavones[22,23,27,28,32,33,36,37,41].

IV. *Type of Mobile Phase*

Both isocratic and gradient mobile phases can be used for HPLC separation of isoflavones; however,

the latter is adopted most frequently as a better separation power can be achieved for the 12 soy isoflavones by a proper control of the polarity[22,23,26,27,31,33,35-39,41]. Isocratic mobile phase can also be employed, but only a few isoflavones could be separated at the same time[28,34,40,42].

V. *Flow Rate of Mobile Phase*

A high flow rate can reduce retention time, while a low flow rate can extend retention time. In most studies, the optimum flow rate of 1-2 mL/min is used for separation of soy isoflavones, yet very low or high flow rates such as 0.2 or 5 mL/min are also employed in some studies[31,38,42].

GC ANALYSIS FOR SOY ISOFLAVONES

I. *Extraction*

A four-step procedure to extract isoflavones from food samples for GC analysis was developed by Mazur *et al*.[43]: (1) rehydration with distilled water, (2) hydrolysis by enzyme and acid, (3) purification by passing through two ion-exchange chromatographic columns and (4) derivatization. Liggins *et al*.[2,44,45] extracted isoflavones from freeze-dried food samples with 80% aqueous methanol, followed by subjecting to enzymatic hydrolysis by sonicating with 5 mL of 0.1 M acetate buffer (pH 5.0) and incubating overnight at 37°C. The hydrolyzed extract was then partitioned with ethyl acetate and derivatized for analysis. An improved low-temperature treatment for aglycone extraction from soybean was developed by Fenner *et al*.[46]. After extraction of soybean seeds (200 mg) with 2.4 mL of 1 M HCl at 98-100°C for 2 h, 9.6 mL of acetonitrile was added and the tubes were stored overnight at -8 to -10°C to facilitate precipitation of interfering compounds. Low temperature extraction method is to prevent the matrix interference, making the overall analysis faster and less expensive, compared to other clean-up methods. For plasma, samples were subjected to hydrolysis by β-glucuronidase in 0.1 M sodium acetate, extracted with diethyl ether, purified in a Sephadex LH-20 column (1 × 3 cm) by eluting with 6 mL of CO_2-saturated methanol and derivatized for analysis[47].

Adlercreutz *et al*.[48] extracted isoflavones and both conjugated and free lignans from human urine employing two different procedures: (1) for extraction of conjugated lignans and isoflavones, urine sample was poured into a Sep-Pak column (Waters, MA, USA), followed by washing with 0.15 mol/L of acetate buffer (pH 3) and eluting with 3 mL of methanol. The eluate was then applied into a DEAE-Sephadex column (Pharmacia Fine Chemicals, Uppsala, Sweden), washed with 4 mL of 70% methanol and 10 mL of 0.2 mol/L acetic acid in 70% methanol and eluted with 10 mL of 0.3 mol/L LiCl in 70% methanol; (2) for extraction of free lignans and isoflavones, urine sample was mixed with 5 mL of buffer mixture, incubated overnight (37°C) and poured into a Sep-Pak column for elution with 3 mL of methanol. The eluate was then applied into a QAE-Sephadex anion-exchange column, followed by washing with 4 mL of methanol and eluting with 7 mL of 0.2 mol/L acetic acid in methanol. The recoveries of daidzein and genistein were 105.5% and 104.9%, respectively; however, this method appears to be complicated and time-consuming as two steps, extraction and purification, are involved. The sample preparation procedures for GC methods along with conditions for separation of soy isoflavones in both food samples and body fluids are briefly outlined in Table 2.

II. *Separation*

Gas chromatographic methods have been adopted to analyze daidzein and genistein in food and biological samples. An isotope dilution GC method with MS detection in selected ion monitoring (SIM) mode was developed by Mazur *et al*.[43], who employed a vitreous silica column (12.5 m × 0.2 mm i.d., 0.25 μm film thickness) to analyze daidzein, genistein, coumestrol and lignans in food samples. The oven temperature was programmed at 100°C for 1 min and increased to 280°C at a rate of 30°C/min, with temperatures of the transfer line, ion source and analyzer at 310, 250 and 250°C, respectively. The detection limit ranged from 2 to 3 μg/100 g, and the coefficient of variation (CV) for the intra-day and inter-day assays were 3.9 and 7.0% for daidzein, respectively, and 3.1 and 10.6% for genistein. Compared to candy bar, lapacho tea and crisp bread, the levels of daidzein and genistein in soy flour were higher and amounted to 67,369 and 96,914 μg/100 g, respectively.

For routine analysis of total isoflavones in foods and vegetables, Liggins *et al*.[2,44,45] developed a GC/MS method with detection by SIM mode. A high degree of sensitivity was achieved by employing a capillary column (15 m of DB1, J&W, Jones Chromatography, Glamorgan, UK) operated isothermally at 320°C and the contents of daidzein and genistein in soy flour were determined to be 1.05 and 1.11 mg/g, respectively, with the inter-assay CV being 2.7 and 4.7%.

Fenner *et al*.[46] developed an improved GC method with FID by subjecting the aglycone extracts of soybean to low-temperature treatment. A SPB-1 fused silica capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness) was used to separate daidzein and genistein, with temperatures of column at 240°C, injector at 300°C and detector at 300°C. The split ratio was 20: 1 and flow rate of helium was 1.4 mL/min. The total separation time of 2 soy isoflavones (daidzein and genistein) was less than 30 min but is still lengthy.

For analysis of lignans and isoflavones in urine samples, Adlercreutz *et al*.[48] developed a GC/MS

Table 2. GC methods for determination of soy isoflavones in body fluids and foods

Extraction	GC column	Separation condition	References
Urine hydrolyzed and then extracted employing a SPE cartridge with 5% methanol and ethyl acetate-acetonitrile (1: 1, v/v), dried and derivatized (pyridine, BSTFA and TMCS).	Dimethylpolysiloxane column (15 m × 0.25 mm i.d., 0.25 μm film thickness, GC^2 chromatography, Manchester, UK)	Oven temperature program: 160°C increased at 10°C/min to 300°C and held for 1 min; SIM/MS	Grace *et al*. (2003)
Food sample mixed with 80% methanol, sonicated (10 min), kept for 1 h, filtered, hydrolyzed and extracted with ethyl acetate for derivatization (pyridine and TBMS).	GC capillary column (15 m of DB1, J & W, Jones Chromatography, Mid Glamorgan, UK)	Isothermally at a temperature of 320°C; MS	Liggins *et al*. (1998)
Food sample hydrolyzed and then extracted by diethyl ether, followed by drying, redissolving in methanol and transferring into a DEAE-Sephadex column for elution with 0.1 M acetic acid in methanol, followed by pouring into a QAE Sephadex column for elution with 0.2 M acetic acid in methanol and then derivatized (pyridine/HMDS/ TMCS, 9: 3: 1, v/v/v).	BP-1 (SGE) vitreous silica column (12.5 m × 0.2 mm i.d., 0.25 μm film thickness)	The temperatures of transfer line, ion source and analyzer are 310, 250 and 250°C respectively, oven temperature program: 100°C kept 1 min and then increased at 30°C/min to 280°C; SIM/MS	Mazur *et al*. (1996)
After hydrolysis, food sample extracted with acetonitrile, centrifuged, supernatant dried and derivatized (pyridine and BSTFA).	SPB-1 fused silica capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness)	Injector temperature: 300°C, oven temperature: 240°C, detector: 300°C; FID	Fenner. (1996)
Plasma hydrolyzed and then extracted with diethyl ether, dried, redissolved in methanol and applied a Sephadex LH-20 column for elution with methanol saturated with carbon dioxide and derivatized (BSTFA).	DB-1 fused silica capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness)	Operated isothermally at 280°C; MS	Morton *et al*. (1994)
Conjugation form extraction Urine extracted by Sep-Pak column with acetate buffer and methanol, purified in a DEAE-Sephadex column with 0.3 mol/L LiCl in 70% methanol as elution solvent, dried and derivatized (pyridine/HMDS/TMCS, 9: 3: 1, v/v/v). Free form extraction After hydrolysis, urine sample was poured into a Sep-Pak column for elution with methanol, followed by applying into a QAE-Sephadex column for elution with 0.2 mol/L acetic acid in methanol and then derivatized (pyridine/ HMDS/TMCS, 9: 3: 1, v/v/v).	BP-1 (SGE) vitreous silica column (12.5 m × 0.2 mm i.d., 0.25 μm film thickness)	The temperatures of transfer line, ion source and analyzer are 310, 250 and 250°C respectively, oven temperature program: 100°C kept 1 min and then increased at 30°C/min to 280°C; MS	Adlercreutz *et al*. (1991)

method to determine daidzein, genistein and equol in human urine by using a BP-1 (SGE) vitreous silica column (12.5 m × 0.2 mm i.d., 0.25 μm film thickness) with oven temperature programmed at 100°C for 1 min and increased to 280°C at a rate of 30°C/min. The temperatures of the transfer line, ion source and analyzer were 310, 250 and 250°C, respectively. The total separation time was within 12 min and the detection limit ranged from 3 to 4 nmol/L. The levels of daidzein, genistein and equol in the urine samples of Finnish omnivorous men varied from 13.3 to 628.0, 21.8 to 1180.0 and 13.9 to 64.3 nmol/24 h, with CV of intra-day and inter-day variation ranging from 0.8 to 15.2% and 4.1 to 13.9%, respectively. A short retention time was achieved by this method, however, it failed to provide adequate resolution for daidzein and genistein.

In a later study, Morton *et al*.[47] used a fused silica capilliary column (30 m × 0.32 mm i.d., 0.25 μm film thickness) operated isothermally at 280°C for analysis of plasma samples collected from postmenopausal Australian women. Helium was used as carrier gas at 1.5 bar and ions were generated at 70 eV using a nominal

accelerating voltage of 4 kV at an ion source temperature of 250°C. The retention times of daidzein and genistein were 7.07 and 8.00 min, respectively, with the mean concentration of daidzein being higher in the plasma of women supplemented with soybean diet (68.3 ng/mL) than with clover sprouts (49.1 ng/mL).

Grace *et al*.[30] quantified isoflavones (daidzein, equol, genistein and glycitein) and lignans in human urine by GC/MS in SIM mode. A GC-1 100% dimethylpolysiloxane column (15 m × 0.25 mm i.d., 0.25 μm film thickness) was used with oven temperature programmed at 160°C initially, increased to 300°C at 10°C/min and maintained for 1 min with a total run time of 15 min. The interface temperature was 200°C, electron ionization was 70 eV and emission current was 150 μA, with the total separation time being less than 15 min. The detection limits of daidzein, equol, genistein and glycitein were 1.3, 1.3, 1.3 and 4.3 ng/mL, respectively, with the levels in urine samples from healthy women being 200.1, 6.1, 102.5 and 34.9 ng/mL.

Invariably, all the GC methods require at least 3 sample preparation steps: hydrolysis, Sep-Pak purification and derivatization, which are time-consuming. Besides, after hydrolysis of sample extract, the total isoflavones are determined based on daidzein and genistein, but not all the soy isoflavones. This limits the application of GC method in isoflavone analysis. Nevertheless, GC method offers detection of total isoflavones in foods and biological samples with high sensitivity.

IMMUNOASSAY ANALYSIS FOR SOY ISOFLAVONES

In addition to GC/MS and HPLC/MS, the immunoassay can be an option in analyzing isoflavones in biological samples. Wang *et al*.[49] developed a time-resolved fluoroimmunoassay (TR-FIA) method to measure daidzein and genistein in human plasma by employing an europium chelate as a label. This method was based on synthesizing two specific antibodies, namely, 4'-*O*-carboxymethyl-daidzein against daidzein and 4'-*O*-carboxymethyl-genistein against genistein, which were used as haptens and conjugated into the carrier protein (BSA) as immunogens for immunization of rabbits. The synthesis of 4'-*O*-carboxymethyl-genistein and -daidzein was carried out by selective alkylation of the phenolic 4'-hydroxyl group of genistein or daidzein using potassium tert-butoxide and ethyl bromoacetate in dimethylformamide, respectively, followed by hydrolysis under acidic conditions. The tracers with the europium chelate were synthesized using the same 4'-*O*-derivative of the isoflavones. After enzymatic hydrolysis and ether extraction, the immunoassay was practiced by using a multilabel counter. In the dissociation enhanced lanthanide fluoroimmunoassay (DELFIA) system, a nonfluorescent chelate was used to bind europium to the analyte. The europium ions were dissociated from the chelates by a solution after the bioaffinity reaction and the lanthanide ions formed a highly fluorescent complex with component in the enhancement solution. After excitation of the fluorophore, the enhanced fluorescence was detected at a fixed time. The detection limits of daidzein and genistein were 1.8 and 3.1 pg/20 μL, respectively. An acceptable limit of precision and accuracy are achieved by immunoassay, which provides a satisfactory performance and is comparable to GC in specificity and sensitivity.

Kohen *et al*.[50] depicted that non-isotopic immunoassay techniques are fast, sensitive and suitable for large scale screening of biological samples derived from epidemiological studies. A TR-FIA method was developed to measure the daidzein concentration in human urine, with a highly daidzein-specific monoclonal antibody (clone 4E4) and a europium-labeled ovalbumin daidzein conjugate being used. The sensitivity of this method is 0.5 ng/mL.

Talbot *et al*.[51] developed a high throughput TR-FIA method for determination of daidzein, genistein and equol in blood and urine. Three specific monoclonal antibodies each specific for genistein, daidzein and equol were used for both blood and urine samples. The detection limit of this method was 3.9 nmol/L for daidzein, 88.8 nmol/L for genistein and 2.2 nmol/L for equol. This outcome indicated that immunoassay offers potential advantages of direct sample analysis, small sample volume, simplicity, speed, low cost, high throughput and automatic equipment. However, a higher urinary phytoestrogen level was reported by TR-FIA than by GC/MS, which is probably due to the purification and derivatization steps to cause isoflavone loss for the latter.

Lapcik *et al*.[52] established a radioimmunoassay (RIA) method to determine daidzein from body fluids, which is based on polyclonal antibodies against daidzein-4'-O-(carboxymethyl)-ether-BSA. The sensitivity of this assay was 0.4 pg/tube. Lapcik *et al*.[53,54] also used a RIA method to identify isoflavones in beer and measure free genistein in human serum. Isoflavones in beer were quantified by two radioimmunoassays, the first one antibody against daidzein/formononetin and the second one against genistein/biochanin A. A total of 26 beer samples were analyzed for isoflavones concentration, with daidzein in beer ranging from 0.08 to 2.5 nmol/L and genistein from 0.169 to 6.74 nmol/L. For free genistein in human serum, two RIA systems for genistein were used, based on polyclonal antibodies against genistein-4'-*O*-(carboxymethyl)-ether-bovine serum albumin and genistein-7-*O*-(carboxymethyl)-ether-bovine serum albumin conjugates, with the sensitivity for genistein being 1.2 and 2.8 pg/tube, respectively. This study implied that immunoassay based on antisera against derivatives differing in the position for construction of the immunogen, which could discriminate between free isoflavones and their different metabolites. The genistein concentrations in 26 Czech omnivore volunteers varied from 0 to 1.24 nmol/L when assayed with the

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G4'-RIA, and from 0 to 1.67 nmol/L when assayed with G7-RIA. Genistein reached a peak in 4 h in serum after consumption of 125 g of cooked soybeans. The G7-antisera may be applicable to screening of genistein in food and plant material as the predominant form of genistein is 7-O-glucoside genistin. Furthermore, this method is sensitive to position-specific derivatives of isoflavones, which should be a useful tool for detailed study of their metabolic fate in body. Compared to RIA, FIA provides the advantages of nonradioisotopic assay such as stability, lack of radiation and no waste problems, and thus should be applicable for routine work. Nevertheless, the enzyme immunoassay (EIA) kit commercially available today can provide a better sensitivity than the conventional RIA and EIA methods. Despite all these merits, the most disadvantage point in immunoassay is the lack of simultaneous evaluation for multi-compounds in one analysis because a very good antibody can recognize only a very special and tiny structure called epitope and bind to only one isoflavone, but cannot identify any other analogs. On the other hand, in case an antibody can recognize all these 12 isoflavones, its specificity will be very poor and it cannot distinguish one isoflavone from the others.

COMPARISON OF SOY ISOFLAVONE ANALYSIS BY HPLC, GC AND IMMUNOASSAY

An ideal analytical method is characterized by high sensitivity, adequate peak resolution, low detection limit, short analysis time and high reproducibility. However, it is often difficult for a single method to achieve all these goals simultaneously. The GC/MS method allows specific and sensitive analysis of soy isoflavones, and reduces solvent cost and solvent disposal. However, most GC/MS methods can only separate a few soy isoflavones (daidzein, genistein and glycitein) and require a time-consuming sample preparation step involving hydrolysis, purification and derivatization prior to analysis. In addition, after hydrolysis of sample extract, the isoflavones are quantified for daidzein and genistein only, but not all soy isoflavones are derivatized with ease. The artifacts generated during derivatization may create significant interference during subsequent GC analysis.

Unlike GC, HPLC methods with gradient solvent system containing an acid as modifier are employed to analyze isoflavones, which are often coupled with UV, DAD or MS for detection. Compared to HPLC/UV or HPLC/DAD, HPLC/MS provides a more rapid and sensitive analysis of soy isoflavones, with detection limit at picogram level. However, Thomas *et al*.[41] pointed out that the free soy isoflavones may pose some pitfalls during HPLC/MS analysis. For instance, glycitein is more labile under high temperature condition during APCI-MS analysis. Also, the phenolic phytoestrogens eluted with acidic solvents do not easily undergo ionization and thus a post-column modification of mobile phase pH is essential. On the other hand, the commonly available detectors like UV or DAD are able to provide adequate sensitivity and resolution for routine analysis. Yet, most HPLC methods fail to separate 12 soy isoflavones in a single run, and, the problems frequently encountered include long retention time, inadequate resolution and complex mobile phase system. Nevertheless, HPLC methods developed by Hsieh *et al*.[37] and Rostagno *et al*.[38] could adequately resolve all the 12 soy isoflavones within a time of 15 and 10 min, respectively, with the former employing a relatively simple mobile phase containing water and acetonitrile.

Comparatively, both GC and HPLC are inappropriate to analyze a large number of samples in a short time. But for immunoassay, it can not only apply to a large number of samples, but also is very versatile, fast and convenient for analysis of soy isoflavones at very low concentration even in complex samples. Additionally, the GC/MS method requires time-consuming sample preparation, while HPLC/MS method needs purification[52]. Also, both methods require expensive instrumentation and are less suitable for epidemiological research. In contrast, the immunoassay is highly specific, sensitive, easy to operate, and possible to analyze some other isoflavones including daidzein and genistein in cells and tissues to improve knowledge in turnover and metabolic pathway of isoflavones. Wang *et al*.[49] concluded that the immunoassay could provide a high-throughput and more sensitive analysis of soy isoflavones than GC or HPLC. However, the immunoassay can analyze only a few isoflavones.

CONCLUSIONS

The GC/MS methods provide not only specific and sensitive analysis of soy isoflavones, but also reduce solvent cost and solvent disposal. However, most GC/MS methods can only separate a few soy isoflavones and require a time-consuming sample preparation step. Similarly, the HPLC methods provide rapid and sensitive analysis of all the 12 soy isoflavones simultaneously. Both GC and HPLC are inappropriate for analysis of a large number of samples at the same time. Conversely, the immunoassay not only provides high throughput, but also are fast and convenient for analysis of soy isoflavones at very low concentration even in complex samples. Nevertheless, only a few isoflavones can be determined by immunoassay methods, and quantitation accuracy may be decreased. Thus, irrespective of nature of the samples, HPLC/UV/MS can provide a convenient and comprehensive determination of all the 12 soy isoflavones, as GC methods are more time-consuming and the number of compounds separated is limited in immunoassay methods.

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