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Original Article

A Method for Determination of Twelve Isoflavones in Soybean by Ultra-Performance Liquid Chromatography

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To establish a method for determining twelve isoflavone compounds by ultra-performance liquid chromatography. A soybean power sample were weighed accurately, followed by extracting with ethanol, then the supernatant was filtered for analysis. The separation was achieved on C_{18} column by gradient elution. The signal was detected with ultraviolet detector in 260 nm. The calibration curves were indicative of high linearity (r^2 > 0.9999) for each analyte in the concentration range of 0.5-25 μg/ml. Limits of detection were 0.126-0.156 μg/ml. The recoveries were obtained by spiking in the range from 93.2 to 107.3%. The inter-day and intra-day precision expressed at coefficient of variation were 0.41-13.07% and 0.40-10.27%, respectively. The method exhibits high sensitivity and repeatability, which can be applied to the determination the amounts of isoflavones in soybean.

Key words: isoflavone, soybean, ultra performance liquid chromatography

Introduction

Soybean, scientific name is *Glycine max Merrial*, belongs to Liguminales order. It is rich in protein and various physiologically active substances in legume seed plants. It is a common food in Asian countries. Isoflavones is a kind of flavonoids. It is a general name for polyphenols is found in soybeans, safflower alfalfa date palm, pomegranate and flaxseed (30, 31, 33). Among them, soybean It is the most commonly known as soy isoflavone (11) which is a secondary metabolite formed during the growth of soybean plants (19). Since its chemical structure is similar to human estrogen (17β-estradiol), it has similar estrogen efficacy. It competes with estrogen receptors for binding, has antioxidant capacity (17, 25), reduces low-density lipoprotein (LDL) (3) in the blood, and is called phytoestrogens (33). Studies have found that soy isoflavones have antioxidant biological activity (5), anti-inflammatory and anti-allergic functions (31). It can ease and improve the symptoms of menopausal discomfort and lipid metabolism (1, 4, 27). Soy isoflavones also control the growth of cancer cells in breast, endometrium, colon, prostate, lung, and skin (2, 22, 29). It protects cardiovascular system by reducing blood fat, which prevents the occurrence of chronic diseases and cardiovascular diseases (2, 9, 27, 28). In recent years, as the isoflavones in soybean have significant effects in the prevention and treatment of many diseases, it has become a hot research topic.

Soy isoflavonoides, naturally existing in soybean, have 12 major components which are divided into two types: the aglycone and glucosidic conjugated. According to the chemical structure of the functional group, soy isoflavones can be divided into 4 categories, 1. The aglycones are daidzein, genistein, and glycitein (Fig. 1.); 2. The β-glucosides are daidzin, genistin, and glycitin (Fig. 2.); 3. Acetyl glucosides are 6″-*O*-acetyldaidzin, 6″-*O*-acetylgenistin, and 6″-*O*-acetylglycitin (Fig. 3.); 4. Malonyl glucosides are 6″-*O*-malonyldaidzin, 6″-*O*-malonylgenistin and 6″-*O*-malonylglycitin (Fig. 3.) (13, 31). In twelve isoflavones, the aglycone account for 2-5% (w/w), and glucosidic conjugate isoflavones account for 95-98% (w/w). Glucosidic conjugate isoflavones of which are mainly daidzin and genistin, 6″-*O*-malonyldaidzi and 6″-*O*-malonylgenistin account for 80-90% (7, 32).

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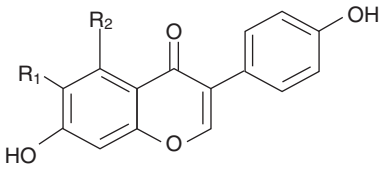
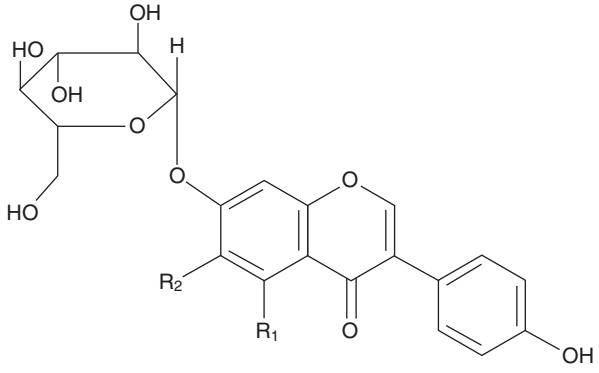


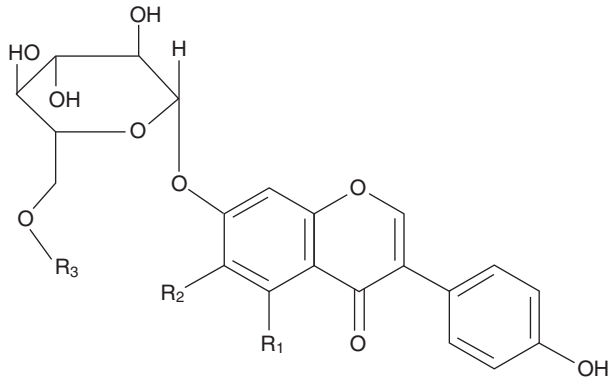
Compounds	R_1	R_2
Daidzein	H	H
Glycitein	H	OCH_3
Genistein	OH	H

Fig. 1. Chemical structure of aglycone isoflavone.



Compounds	R_1	R_2
Daidzin	H	H
Glycitin	H	OCH_3
Genistin	OH	H

Fig. 2. Chemical structure of β-glucosides.



Compounds	R_1	R_2	R_3
6″-*O*-Acetyldaidzin	H	H	$COCH_3$
6″-*O*-Acetylglycitin	H	OCH_3	$COCH_3$
6″-*O*-Acetylgenistin	OH	H	$COCH_3$
6″-*O*-Malonyldaidzin	H	H	$COCH_2COOH$
6″-*O*-Malonylglycitin	H	OCH_3	$COCH_2COOH$
6″-*O*-Malonylgenistin	OH	H	$COCH_2COOH$

Fig. 3. Chemical structures of acetyl glucosides and malonyl glucosides.

Relevant literature on isoflavone detection methods includes the detection of isoflavone and its metabolites in human urine by gas chromatography (GC) (15), the use of gas chromatography-mass spectrometry (GC-MS) detection of isoflavone content in urine (21), detection of daidzein and genistein content in food by GS-MS (20). Liquid chromatography mass spectrometry (LC-MS) and ultraviolet (UV) spectrophotometry were also used to detect the isoflavone content in soybeans (6, 10, 18, 26). In the above analytical methods, it is simple to operate with UV spectrophotometry but the specificity is poor. The GC and GC-MS have a small sample injection volume, good selectivity and sensitivity. But sample needs to be hydrolyzed or derivatized for glucosidic conjugated isoflavones detection. LC-MS is easy to identify and quantify, but the instrument is expensive and high maintenance cost. Because high performance liquid chromatography (HPLC) has many advantages of various sample detection, such as a wide range, high separation efficiency and sensitivity, and does not require complicated preprocessing steps (19, 26), it is widely used for isoflavone analysis (8, 14, 16, 24, 30, 33). Many studies using HPLC to analyze soy isoflavones, most of them only test aglycone (daizein, genistein, glycitein) and β-glucosides (daizin, genistin, glycitin) separately, and there are fewer studies on the determination of 12 isoflavones at the same time. According to the research reports, some isoflavones have similar characteristics. In order to obtain good chromatogram, the run time need more than 30 minutes. Therefore, it also consumes a large amount of organic solvents and increases the inspection cost. In order to reduce the analytical time and improve the resolution of chromatogram, the ultra performance liquid chromatography (UPLC) was used to separate 12 isoflavones at the same time.

Materials and Methods

Equipments

ACQUTY UPLC system includes a binary solvent manager, sample manager, and photodiode array detector (PDA) from Waters (Millford, MA, USA). The analysis software is Empower 2 software. The analytical column was ACQUITY BEH C_{18} (2.1 × 100 mm, particle size 1.7 μm) purchased from Waters. Electronic balance AB265-S/FACT PL-3002 was purchased from Mettler Toledo (Columbus,

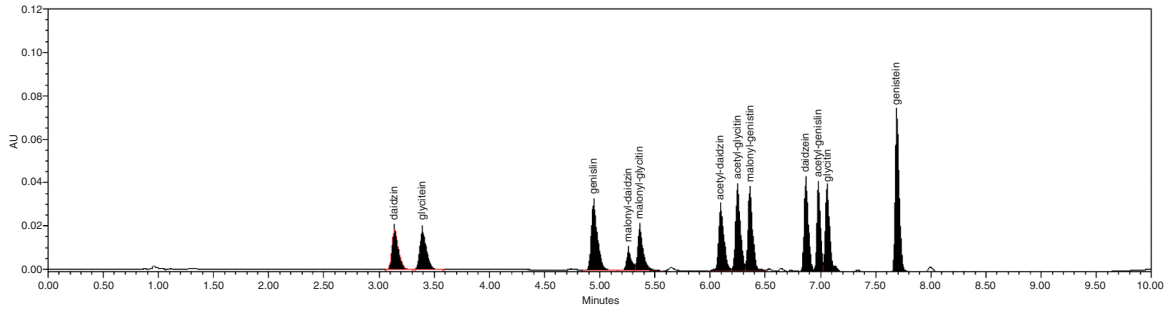


Fig. 4. UPLC chromatograms of 12 isoflavone standards at a concentration of 5 μg/ml.

Ohio, USA).

Chemicals and Reagents

Daidzein ($C_{15}H_{10}O_4$, purity ≧ 98%), genistein ($C_{15}H_{10}O_5$, purity ≧ 98%), glycitein ($C_{16}H_{12}O_5$, purity ≧ 98%), daidzin, ($C_{21}H_{20}O_9$, purity ≧ 98%), genistin ($C_{21}H_{20}O_{10}$, purity ≧ 98%), and glycitin ($C_{22}H_{22}O_{10}$, purity ≧ 98%) were purchased from Chem Faces (Hubei, PRC). Acetyldaidzin (6″-*O*-Acetyldaidzin $C_{23}H_{22}O_{10}$, purity:> 95%), acetylgenistin (6″-*O*-Acetylgenistin $C_{23}H_{22}O_{11}$, purity > 95%), acetylglycitin (6″-*O*-Acetylglycitin $C_{24}H_{24}O_{11}$, purity 98.8%), malonyldaidzin (6″-*O*-Malonyldaidzin $C_{24}H_{22}O_{12}$, purity 99.6 %), and malonylgenistin (6″-*O*-Malonylgenistin $C_{24}H_{22}O_{13}$, purity 92.6%) were purchased from Carbosynth (Newbury, Berkshire, UK). Malonylglycitin (6″-*O*-Malonylglycitin $C_{25}H_{24}O_{13}$, Purity 99.1%) was purchased from ACE Biolabs (Hsinchu, ROC). Dimethyl sulfoxide ($(CH_3)_2SO$), ethanol (CH_3CH_2OH, LC grade), acetonitrile (CH_3CN, LC grade), and phosphoric acid (H_3PO_4, 85%) were purchased from MERCK (Darmstadt, Germany). Deionized water (18.2 ΩM) was processed by the microporous MILLI-Q® Direct 8 ultrapure water system.

Sample Preparation

The soybean sample was ground to fine powder and pass through 20 mesh stainless steel sieve. A soybean powder sample was weighed at 1 g and placed in a 50 ml polypropylene centrifuge tube. Subsequently, 15 ml of 70% ethanol solution was added and homogenized for 30 min by shaker. The sample was then centrifuged at a speed of 3500 rpm for 5 min. The supernatant was transferred to another centrifuge tube. A volume of 15 ml of 70% ethanol solution was added to original centrifuge tube and repeat the above procedure. The two supernatants were combined and mix well. Finally the extraction was filtered by passing through a 0.22 μm PTFE filter and was then used for HPLC analysis.

Standard Calibration Curve

Each of 12 isoflavones with 1000 μg/ml was prepared by dissolving 1 mg standard in 1 ml dimethyl sulfoxide to be a stock solution and was stored in a brown glass bottle at 4°C. The stock solutions of 12 isoflavones were mixed and diluted with 40% acetonitrile to a series of concentration 0.5, 1, 5, 10, and 25 μg/ml. According to the concentration versus corresponding area, the linear regression equation $y = ax + b$ was obtained.

UPLC Conditions

UPLC separation of 12 isoflavones was performed on ACQUITY BEH C_{18} column (2.1 × 100 mm, 1.7 μm particle size). The UV detection wavelength is 260 nm. Gradient elution was performed by using mobile phase A (0.05% phosphoric acid) and mobile phase B (acetonitrile). The flow rate was 0.25 ml/min and the following gradient program was applied: The initial ratio of mobile phase A was 87% and B was 13%. The gradient elution procedure was started after 2 min of equilibration, and then mobile phase B was linearly increased to 17% in 3 min, to 25% in 5 min, to 47% in 7 min and held 1 min. Finally, mobile phase B was decreased to 13% in 9 min and held 1 min, for a total run time of 10 min. Injection volume was 1 μl for standard or sample.

Results

The study used acetonitrile and formic acid solutions as mobile phases because of the advantages of simple composition and easy preparation. The 12 isoflavone standards could be successfully separated within 10 minutes under these analysis conditions. The chromatographic results are shown in Fig. 4.

The total soybean isoflavone content generally included four types of aglycone, β-glycosides,

Table 1. Isoflavones linear equation, LOD and LOQ of aglycone and β-glucosides detected by UPLC/PDA with 260 nm.

Isoflavones	Calibration data		LOD (mg/ml)	LOQ (mg/ml)
	Equation	r^2		
daidzin	14008.65x + 537.11	0.99998	0.135	0.450
glycitein	14250.38x + 384.51	0.99998	0.156	0.521
genistin	21271.22x + 642.67	0.99998	0.126	0.418
daidzein	21198.11x + 550.39	0.99998	0.137	0.458
glycitin	13624.68x + 118.00	0.99995	0.150	0.500
genistein	30695.41x + 873.28	0.99998	0.149	0.497

LOD: limit of detection; LOQ: limit of quantification.

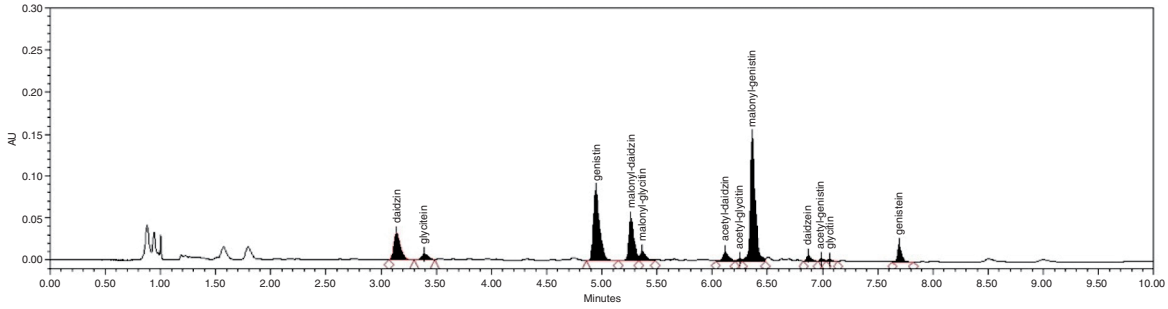


Fig. 5. Chromatogram of 12 isoflavones extracted from soybean sample.

acetyl glucosides and malonyl glucosides. Among them, acetyl glucosides and malonyl glucosides were calculated indirectly from the standard curve of glycoside. Therefore, the acetyl glucosides and malonyl glucosides isoflavone standards were only used in this experiment. In order to identify whether the sample contained acetyl glucosides and malonyl glucosides isoflavones, the standard curve was made only for six isoflavone standards such as aglycone and β-glucosides. Diluted isoflavone standards to the calibration curve concentration range from 0.5 μg/ml to 25 μg/ml, and used the concentration as the horizontal axis and the integrated area as the vertical axis to make a standard curve. The regression equations are shown in Table 1. The square of correlation coefficient was above 0.99995, all of which had a good linear relationship. The LOD evaluation of isoflavones was the concentration corresponding to the blank signal value plus 3 times the standard deviation of the blank signal value. The LOQ was the concentration corresponding to the blank signal value plus 10 times the standard deviation of the blank signal value. The concentrations were 0.126-0.156 mg/L for LOD, 0.418-0.521 mg/l for LOQ, respectively. According to the sample extraction step, the extracted test solution was analyzed by UPLC, and the chromatogram could be obtained (Fig. 5.).

The accuracy of the method was evaluated by the spiked recovery experiment. To the soy sample test solution, standard daidzein, genistein, daidzin, genistin and glycitin were 0.5, 1 and 2 μg/ml, and glycitein was 1, 2 and 5 μg/ml. Each concentration was repeated 5 times and the average was calculated. The average recovery of the six soy isoflavones was 93.2-107.3% (Table 2). Intra-day and inter-day analysis were used to evaluate repeatability and reproducibility. As Table 3, the coefficient of variations (CV) were 0.43-6.42% for repeatability, 0.92-12.78% for reproducibility, respectively. The repeatability and reproducibility test results can prove that the method has good precision.

Discussion

Most of the analysis methods for soybean isoflavones use LC. Because similar chemical structure and properties of soybean isoflavones, three mobile phase including formic acid solution, methanol and acetonitrile can present a good separation results in some studies. In LC, acetonitrile has stronger elu-

Table 2. Average recovery of the developed method at four different spiked concentrations for aglycone and β-glucosides isoflavone in soybean sample.

Isoflavones	Average recovery ± CV%			
	0.5 μg/ml	1 μg/ml	2 μg/ml	5 μg/ml
daidzein	93.5 ± 3.2	95.9 ± 1.6	95.4 ± 1.5	–
genistein	95.6 ± 1.3	96.1 ± 0.9	95.4 ± 0.8	–
daidzin	104.1 ± 6.5	100.5 ± 4.6	96.8 ± 3.2	–
genistin	101.8 ± 11.4	106.3 ± 6.7	102.1 ± 5.3	–
glycitin	102.3 ± 6.8	104.2 ± 2.7	100.2 ± 1.5	–
glycitein	–	107.3 ± 2.4	102.6 ± 1.0	93.2 ± 7.4

n = 5

Table 3. Repeatability and reproducibility of the developed method at three different spiked concentrations for aglycone and β-glucosides isoflavone in soybean sample.

Isoflavones	Concentration, μg/ml	Intra-day, CV%[a]	Inter-day, CV%[b]
daidzein	0.5	1.13	2.27
	1	1.14	1.84
	2	0.61	6.47
genistein	0.5	0.80	0.97
	1	0.69	0.92
	2	0.59	4.72
daidzin	0.5	4.05	8.09
	1	1.20	1.77
	2	1.82	4.31
genistin	0.5	6.42	12.78
	1	4.80	6.64
	2	3.52	6.20
glycitin	0.5	5.20	7.62
	1	0.43	2.81
	2	1.24	3.56
glycitein	1	1.37	2.09
	2	0.82	1.12
	5	0.93	7.45

[a]: n = 5; [b]: n = 3

tion ability than methanol, thus using acetonitrile as mobile phase can obtain better separation results for isoflavone analysis (10). In Yanaka K. and Kim J.A. studies, acetonitrile and acetic acid as mobile phases can separate four types of soybean isoflavones simultaneously (16, 33).

The purpose of the sample preparation is to extract the target compounds from the matrix. In the extraction step, organic solvents are often used as extracted solvent. Although this extraction solvent can improve the extraction rate of the analyte, many co-extracted components also be extracted, therefore, the purification procedure are usually required to eliminate the matrix such as using solid phase

Table 4. Isoflavone content in different soybean species.

unit: μg/g

Species	β-glucosides			Malonyl glucosides			Acetyl glucosides			Aglycone			Total isoflavone
	DIN[a]	GLY[b]	GIN[c]	MDIN[d]	MGLY[e]	MGIN[f]	AcDIN[g]	AcGLY[h]	AcGIN[i]	DEIN[j]	GLEIN[k]	GEIN[l]	
A	330	15	360	1361	96	1576	171	24	12	28	60	25	4058
B	58	23	126	169	153	426	53	13	7	0	102	17	1149
C	366	9	381	1590	95	1720	197	12	4	22	62	20	4479
D	344	16	442	1387	168	1965	220	21	14	33	92	32	4736
E	276	20	433	370	64	709	82	8	17	42	70	55	2146
F	724	17	1115	1040	55	1642	199	16	53	136	75	161	5232
G	269	13	325	628	63	782	85	6	4	22	48	20	2268
H	342	10	419	1680	149	2002	228	20	11	24	72	21	4980
I	192	7	398	224	ND	482	55	4	11	25	ND	33	1432
J	68	15	104	268	145	490	53	21	4	16	77	19	1279

ND: not detected; a. daidzin; b. glycitin; c. genistin; d. malonyldaidzin; e. malonylglycitin; f. malonylgenistin; g. acetyldaidizin; h. acetylglycitin; i. acetylgenistin; j. daidzein; k. glycitein; l. genistein.

extraction (SPE) (24). These purification procedure usually increases analysis time and elevates experimental costs. The sample preparation refers to the research report, using 70% ethanol solution as the extracted solvent (23, 33).

The content of isoflavones in soybean are affected by environmental conditions such as temperature, moisture, and light, as well as different soybean species, planting places, or production date. There are 12 naturally soy isoflavones, of which the highest content of glycones and malonyl glucosides are the major type, accounting for more than 90%, but β-glucosides accounts for less than 10% (7, 13). Most soybeans in the United States and Japan have an isoflavone content of about 1411-4216 μg/g. A small number of species have isoflavone content of less than 1000 μg/g. Basically, French soybeans have the highest isoflavone content in the range of 3714-9540 μg/g (14). This study also actually measured the isoflavone content of different species of soybeans. Ten different species of soybeans were selected, and each sample was measured three times. The content of isoflavones was calculated according to the standard method of the Ministry of Health and Welfare Food and Drug Management Plan (23). The aglycone and β-glucosides isoflavone contents were calculated from aglycone and β-glucosides standard curves. The inverse linear equation slope of aglycone and β-glucosides were taken as the corresponding reaction coefficient of acetyl glucosides and malonyl glucosides calculation. Calculation of the isoflavone content in 10 different species of soybeans, which total isoflavones content were in the range of 1149-5232 μg/g, was shown in Table 4. In addition, the measured types of isoflavones were the highest in β-glucosides and malonyl glucosides and the results were consistent with literature (12, 16, 31).

Conclusion

This experiment established the simultaneous determination of 12 soy isoflavones in soybeans by UPLC. The sample was extracted with 70% ethanol solution through a simple procedure and separated of simple mobile phase with C_{18} column. Twelve soy isoflavones were separated by GC, and had good separation effective. In this method, the accuracy and precision of the sample recovery were evaluated well. At the same time, different species of soybeans were detected, and the results were as similar as related literature reports. Therefore, the method of sample extraction, small volume sampling, and the short time analysis can be used as a method for isoflavone content detection in soybean.

Acknowledgements

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Conflicts of Interest

The authors declare no conflict of interest.

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