ELSEVIER

Journal of Nutritional Biochemistry 15 (2004) 452–460

THE JOURNAL OF Nutritional Biochemistry

Determination of phytoestrogens in traditional medicinal herbs using gas chromatography–mass spectrometry

Sang Hee Lee[a,b], Byung Hwa Jung[a], Sun Yeou Kim[b], Bong Chul Chung[a,*]

[a]*Bioanalysis and Biotransformation Research Center, Korea Institute of Science and Technology, P. O. Box 131, Cheongryang, Seoul, 130-650, South Korea*

[b]*Graduate School of East-West Medical Science, Kyung Hee University, Seoul 151-742, South Korea*

Received 19 February 2003; received in revised form 28 November 2003; accepted 4 January 2004

Abstract

Quantitative analytical methods for the 17 phytoestrogens containing isoflavonoids, lignans, and mycoestrogens in herbs were developed, and the amount of phytoestrogens was determined in 22 traditional medicinal herbs. The focus of this study was to simplify the purification procedure for removing many kinds of interferences in herbs, and to select adequate derivatization reagent for getting desirable selectivity and sensitivity in the quantitative determination of phytoestrogens. To satisfy these goals, we performed a solid-phase extraction with Oasis HLB cartridges following enzymatic and acidic hydrolysis, and we used the mixture of MSTFA/NH_4I/DTE (1000:4:5, v/w/w) to form TMS derivatives of phytoestrogens. Overall recovery was more than 84% in all of the phytoestrogens, and the limit of quantification for phytoestrogens in herbs were set at 0.2 μg/g. Coefficient of variation percentages were in the range of 0.18–15.68% (within-day) and 0.23–16.61% (day-to-day), respectively. Most of the isoflavonoids and lignans were found in all of the herbs, but mycoestrogens were not detected at all. The *Leguminosae* family proved to be the richest source of isoflavonoids. Lignans such as enterodiol and enterolactone were detected at low concentration in most of the herbs. These results indicate that this assay is accurate and reliable for the determination of phytoestrogens in herbs. Also, information regarding the phytoestrogen contents in traditional medicinal herbs is useful in the prevention and treatment of chronic diseases such as cancer, osteoporosis, dementia, and cardiovascular disease.

Keywords: Gas chromatography–mass spectrometry (GC-MS); Phytoestrogens; Traditional medicinal herb; Isoflavonoids; Lignans

1. Introduction

Phytoestrogens are plant-derived, nonsteroidal compounds that possess estrogen-like biological activity [1]. The dietary consumption of phytoestrogens has been associated with a lower incidence of breast, colon, and prostate cancer [2,3] in Asia, where phytoestrogen intake is high compared with that in western countries [4,5]. Recently many researchers have reported that phytoestrogen intake plays a role in preventing the development of some chronic diseases such as age-related bone loss [6,7], memory loss [8], dementia [9], and cardiovascular disease [10,11].

Recent work has also shown that phytoestrogens decreased the tissue level of 8-hydroxy-2′-deoxyguanosine, which is a marker for oxidative DNA damage. The results indicate that antioxidant properties of phytoestrogens are considered to be responsible in part for their protective effects against oxidative insults [12,13]. Because of those beneficial effects of phytoestrogens, many researchers have been interested in the possibility of phytoestrogens for the treatment of many diseases and tried to identify plants or food that are rich in phytoestrogens. To determine the content of phytoestrogens in plants or foods, many quantitative analyses have been performed [14–18]. However, most methods did not have sufficient sensitivity and selectivity for the determination of phytoestrogens at low concentrations. In addition, the reported extraction and purification steps for phytoestrogens in herbs [20] were so complex and dependent on individual experimental skill that they led to significant losses of phytoestrogens. Therefore, we tried to develop a simpler and more sensitive analytical method for the determination of phytoestrogens in herbs. In this study we especially focused on the simplification of a column-chromatography procedure to remove impurities and to concentrate phytoestrogens, and tried to improve sensitivity

* Corresponding author. Tel.: 82-2-958-5067; fax: 82-2-958-5059.
E-mail address: bcc0319@kist.re.kr (B.C. Chung).

0955-2863/04/$ – see front matter
doi:10.1016/j.nutbio.2004.01.007

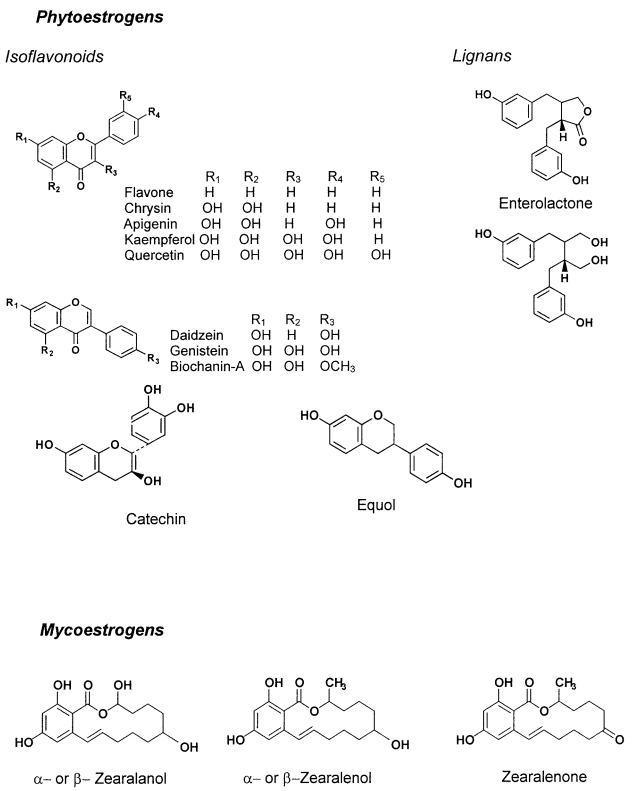

Fig. 1. Chemical structures of phytoestrogens, isoflavonoids, flavone, chrysin, apigenin, kaempferol, quercetin, daidzein, genistein, biochanin-A, catechin, equol and lignans, enterolactone, enterodiol and mycoestrogens, α- or β-zearalanol, α- or β-zearalenol, zearalanone.

from the changing of derivatization reagents. We then chose 22 traditional medicinal herbs that have been known to be very effective in the prevention and treatment of chronic diseases such as cancer, osteoporosis, dementia, and cardiovascular disease in Korea, and determined the content of phytoestrogens in those herbs with this developed method.

2. Methods and materials

2.1. Materials

All phytoestrogens, flavone, chrysin, apigenin, quercetin, kaempferol, catechin, equol, daidzein, genistein, biochanin-A, enterodiol, enterolactone, α-zearalanol, β-zearalanol, zearalanone, α-zearalenol, and β-zearalenol (Fig. 1) were obtained from Sigma Chemical Co. (St. Louis, MO). 17β-Estradiol-(d_4-2,4,16,16-2H-1,3,5(10)-estratrien-3,17 β-diol, internal standard (IS)) was purchased from MSD Isotope (Montreal, PQ, Canada). N-methyl-N-trifluorotrimethylsilyl acetamide (MSTFA), ammonium iodide (NH_4I), and dithioerythritol (DTE) were purchased from Aldrich Co. (Milwaukee, WI). CH_3COONa, CH_3COOH and HCl were purchased from Junsei Chemical Co. (Tokyo, Japan). β-Glucuronidase/aryl sulfatase from *Helix pomatia* was supplied by Boeringer-Mannheim (Mannheim, Germany). Diethyl ether and methanol were supplied by J.T. Baker Co. (Phillipsburg, NJ). Diethyl ether was distilled from calcium hydride powder just before using. All chemicals and solvents were of the highest grade that was commercially available.

Each stock solution of the standards was prepared by concentration of 1 g/L in methanol. By diluting in methanol, each working standard solution was made up at a varied concentration in the range of 1 to 100 mg/L, and the IS solution was prepared at a concentration of 10 mg/L.

2.2. Preparation of traditional medicinal herbs

The traditional medicinal herbs examined were as follows: *Angelicae dahuricae radix, Bupleuri radix, Clematidis radix, Salviae miltiorrhizae radix, Cimicifugae rhizoma, Angelicae gigantis radix, Scutellariae radix, Sophorae radix, Schizandrae fructus, Acanthopanacis cortex, Epimedii herba, Atractylodis macrocephalae, Anemarrhenae rhizoma, Asari radix, Ponciri fructus, Magnoliae cortex, Cervi cornu pantotrichum* (from New Zealand and Soviet Russia), *Gardeniae fructus, Puerariae radix, Cordyceps,* and *Paeoniae radix lba.* All traditional drided medicinal herbs (500 g) were extracted with a mixture of water and methanol (15:85) under a sonicator (25°C) for 1 hour (3 times), and the filtered MeOH extract was evaporated under vacuum [19].

2.3. Enzymatic hydrolysis

Dried power of the 85% methanol extraction (25 mg) of each herb were taken into a glass test tube and 10 μL of a stock solution of 17β-estradiol-d_4 (10 mg/L) was added into an internal standard. A 1-mL quantity of a 0.2-mol/L acetate buffer (pH 5.2) and 100 μL of β-glucuronidase/aryl sulfatase from *Helix pomatia* (β-glucuronidase activity was 5.5 U/mL at 39°C, and aryl sulfatase activity was 2.6 U/mL at 38°C) were also added for enzymatic hydrolysis. Before hydrolysis, 100 μL of an aqueous solution of ascorbic acid (10 mg/mL) was added to prevent the oxidation of catechol phytoestrogens. The mixture was heated at 55°C for 3 hours to hydrolyze the glycosides of isoflavonoids. After slight cooling at room temperature, the samples were extracted with 5 mL of diethyl ether by mechanical shaking for 10 minutes and centrifuged at 2400 rpm for 5 minutes. The separation between organic and aqueous phases was achieved by freezing. The separated organic layer was evaporated under a gentle stream of nitrogen, and the extraction procedures were repeated twice.

2.4. Acid hydrolysis

To remove the glycosides of lignans, we hydrolyzed the aqueous phase again by adding 50 μL of 6 mol/L hydrochloric acid, and incubating it at 100°C for 2.5 hours. After

Table 1
GC-MS retention times selected ions, calibration range, and linearity of standards phytoestrogens

Compound	Ions Selected (m/z)*	Relative Retention Time (min)	Calibration Range (μg/g)	Linearity (r^2)
Flavone	222, 194, 165	0.88	100–2000	0.9842
Chrysin	398, 383, 311	1.08	4–200	0.968
Apigenin	486, 471, 399	1.56	10–120	0.9834
Kaempferol	574, 559, 487	1.49	20–300	0.9855
Quercetin	662, 647, 559	1.61	4–200	0.9709
Daidzein	398, 383, 355	1.33	40–2400	0.9974
Genistein	486, 471, 399	1.35	40–800	0.986
Biochanin-A	428, 413, 370	1.26	40–800	0.9795
Enterolactone	442, 427, 180	1.19	5–300	0.9988
Enterodiol	500, 410, 180	1.02	3–40	0.9974
Catechin	650, 560, 368	1.26	4–160	0.9548
Equol	386, 371, 192	0.88	0.6–20	0.9863
α-Zearalenol	536, 431, 305	1.28	2–30	0.9878
β-Zearalenol	536, 398, 305	1.31	2–30	0.9872
α-Zearalanol	538, 523, 433	1.20	2–30	0.9901
β-Zearalanol	538, 523, 433	1.22	2–30	0.99
Zearalanone	534, 519, 305	1.24	2–30	0.9678
17β-Estradiol-d_4	420	1		

* Quantitative ions are underlined.

cooling, it was extracted with 5 mL of diethyl ether as described above.

2.5. Solid-phase extraction

The Oasis HLB extraction cartridge (60 mg; Waters Co., Milford, MA), which was placed in a device fitted with a small vacuum pump and a waste receiver, was subsequently preconditioned with methanol and water (1 mL each) at a flow rate of 1.5–2 mL/min. The residue obtained from enzymatic and acid hydrolysis was dissolved with 500 μL of methanol and slowly loaded into the cartridge. The cartridge was then washed with 1 mL of water and eluted with 5 mL of methanol (2.5 mL, twice) at a flow rate of 0.8–1 mL/min. The eluted methanol was evaporated to dryness under a gentle stream of nitrogen and dried in a vacuum desiccator over P_2O_5/KOH for 30 minutes before derivatization.

2.6. Derivatization

The dried residue was reacted with MSTFA/NH_4I/DTE (50 μL, 1000:4:5, v/w/w) by heating at 60°C for 15 minutes to form a TMS derivative before analyzing with gas chromatography–mass spectrometry (GC-MS).

2.7. GC-MS

GC-MS analyses were performed with a Hewlett-Packard model 6890 Plus gas chromatograph interfaced to a Hewlett-Packard model 5970B MSD (Hewlett-Packard, Andover, MA). An Ultra-2 (SE-54 bonded phase) fused-silica capillary column (25 m, length × 0.2 mm, ID, 0.33 μm, film thickness; Agilent) was used. The electron energy was 70 EV and the ion source temperature was 230°C. Each sample (2 μL) was injected in split mode and the split ratio was 5:1. The oven temperature was programmed as follows: the initial temperature was at 160°C; it was ramped to 270°C at a rate of 10°C/min and held for 10 min. Finally, it was ramped to 315°C at 5°C/min and held for 2 min. The carrier gas was helium, and the column flow was 0.4 mL/min at 160°C.

We analyzed all phytoestrogens in the selected ion monitoring (SIM) mode in mass spectrometry. We achieved the peak identification by comparing the retention times and matching the area ratios of three characteristic ions of each compound. The characteristic ions for each phytoestrogens in SIM mode are listed in Table 1.

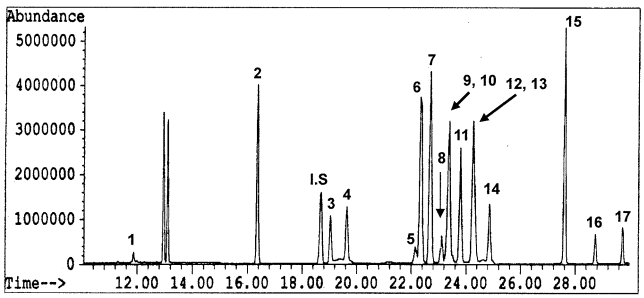

Fig. 2. Total ion chromatogram of phytoestrogens in SIM mode in GC-MS. 1 = flavone; 2 = equol; 3 = enterodiol; 4 = chrysin; 5 = enterolactone; 6 = α-zearalenol; 7 = β-zearalenol; 8 = zearalanone; 9 = biochanin-A; 10 = catechin; 11 = α-zearalanol; 12 = daidzein; 13 = β-zearalanol; 14 = genistein; 15 = kaempferol; 16 = apigenin; 17 = quercetin; IS = 17β-estradiol-d_4.

Table 2
Within-day variations of phytoestrogens

Compound					
Flavone					
Concentration added (μg/g)	100	200	500	1000	2000
Concentration founded (Mean ± SD, μg/g)	102.91 ± 1.15	201.70 ± 2.43	502.22 ± 4.78	991.47 ± 1.66	2003.61 ± 17.75
CV %	1.12	1.21	0.95	0.17	0.89
Chrysin					
Concentration added (μg/g)	4	20	50	120	200
Concentration founded (Mean ± SD, μg/g)	3.70 ± 0.16	20.97 ± 1.63	49.36 ± 2.25	118.45 ± 3.00	204.77 ± 0.10
CV %	4.20	7.78	4.57	2.53	0.05
Apigenin					
Concentration added (μg/g)	10	20	30	50	120
Concentration founded (Mean ± SD, μg/g)	10.50 ± 1.63	20.54 ± 0.37	29.09 ± 0.39	51.79 ± 3.19	120.96 ± 1.63
CV %	15.50	1.78	1.34	6.17	1.35
Kaempferol					
Concentration added (μg/g)	20	40	200	300	
Concentration founded (Mean ± SD, μg/g)	19.64 ± 2.00	38.80 ± 0.46	202.42 ± 1.19	302.54 ± 4.12	
CV %	10.19	1.19	0.59	1.36	
Quercetin					
Concentration added (μg/g)	4	20	50	100	200
Concentration founded (Mean ± SD, μg/g)	4.75 ± 0.28	20.76 ± 0.49	48.23 ± 4.08	97.74 ± 2.28	203.42 ± 1.11
CV %	5.99	2.38	8.45	2.33	0.55
Daidzein					
Concentration added (μg/g)	40	200	400	1200	2400
Concentration founded (Mean ± SD, μg/g)	43.65 ± 2.22	211.96 ± 16.91	386.00 ± 8.90	1194.39 ± 11.09	2383.25 ± 18.07
CV %	5.09	7.98	2.30	0.93	0.76
Genistein					
Concentration added (μg/g)	40	80	200	400	800
Concentration founded (Mean ± SD, μg/g)	40.28 ± 6.31	79.41 ± 6.02	202.51 ± 5.18	395.35 ± 3.48	795.84 ± 7.76
CV %	15.68	7.58	2.56	0.88	0.97
Biochanin-A					
Concentration added (μg/g)	40	120	200	400	800
Concentration founded (Mean ± SD, μg/g)	40.79 ± 5.98	116.42 ± 3.94	200.35 ± 5.13	396.98 ± 4.17	801.97 ± 4.72
CV %	14.65	3.39	2.56	1.05	0.59
Enterolactone					
Concentration added (μg/g)	5	28	50	120	300
Concentration founded (Mean ± SD, μg/g)	5.73 ± 0.74	27.64 ± 0.53	50.41 ± 1.60	119.58 ± 2.33	300.40 ± 2.17
CV %	12.97	1.92	3.17	1.94	0.72
Enterodiol					
Concentration added (μg/g)	3	5	10	20	40
Concentration founded (Mean ± SD, μg/g)	2.99 ± 0.10	5.02 ± 0.10	9.97 ± 0.23	19.85 ± 0.34	40.15 ± 0.10
CV %	3.28	2.08	2.35	1.74	0.25
Catechin					
Concentration added (μg/g)	4	20	50	160	
Concentration founded (Mean ± SD, μg/g)	3.96 ± 0.17	19.16 ± 0.49	48.73 ± 0.18	162.12 ± 0.29	
CV %	4.29	2.56	0.36	0.18	
Equol					
Concentration added (μg/g)	1	5	10	15	20
Concentration founded (Mean ± SD, μg/g)	1.03 ± 0.08	4.82 ± 0.03	10.05 ± 0.29	14.91 ± 0.17	19.83 ± 0.14
CV %	7.80	0.69	2.84	1.11	0.73
α-Zearalanol					
Concentration added (μg/g)	2	4	10	20	30

Continued

Table 2
Continued

Compound					
Concentration founded (Mean ± SD, μg/g)	2.08 ± 0.15	3.99 ± 0.02	9.95 ± 0.05	19.9 ± 0.48	29.95 ± 0.51
CV %	7.33	0.46	0.49	2.43	1.71
β-Zearalanol					
Concentration added (μg/g)	2	4	10	20	30
Concentration founded (Mean ± SD, μg/g)	1.97 ± 0.21	3.99 ± 0.02	10.01 ± 0.21	19.88 ± 0.33	29.89 ± 0.74
CV %	10.75	0.46	2.14	1.66	2.48
α-Zearalenol					
Concentration added (μg/g)	2	4	10	20	30
Concentration founded (Mean ± SD, μg/g)	1.88 ± 0.12	4.25 ± 0.42	10.08 ± 0.09	20.14 ± 0.18	30.11 ± 0.40
CV %	6.50	9.94	0.88	0.89	1.32
β-Zearalenol					
Concentration added (μg/g)	2	4	10	20	30
Concentration founded (Mean ± SD, μg/g)	1.80 ± 0.06	4.55 ± 0.04	10.11 ± 0.25	19.99 ± 0.22	30.23 ± 0.21
CV %	3.58	0.93	2.43	1.12	0.70
Zearalenone					
Concentration added (μg/g)	2	4	10	20	30
Concentration founded (Mean ± SD, μg/g)	1.98 ± 0.07	4.18 ± 0.15	9.87 ± 0.16	19.76 ± 0.82	29.79 ± 0.21
CV %	3.54	3.68	1.60	4.13	0.72

2.8. Validation

Repeatability (within-day variations) studies were carried out on each containing different levels of phytoestrogens. Measurements were repeated three times on the same day. Also, various amounts of phytoestrogens, which has the same concentrations as the test for within-day were analyzed for reproducibility (day-to-day variations) tests. Phytoestrogen contents were determined in triplicate on 3 different days.

3. Results

3.1. Extraction and validation of analytical method for phytoestrogens

The solid phase extraction with Oasis HLB cartridge achieved more than 80% recovery in all phytoestrogens. It was much more convenient to treat and to elute solvents; thus this procedure saved a lot of time to analyze samples. It also removed interference effectively so we could get clear chromatograms in GC-MS analysis (Fig. 2). The derivatization with the mixture of MSTFA/NH_4I/DTE improved the detection limits of phytoestrogens (0.02–0.4 μg/100 g) in GC-MS analysis about 10–100-fold compared with previous reports (2–3 μg/100 g) [20]. The results of validation in quantitative analysis are summarized in Tables 2 and 3. The coefficient of variation (CV) percentages of within-day and day-to-day variations were less than 15.68% and 16.61%, respectively, in all phytoestrogens. These results show that this analytical method has a good recovery, accuracy, and reproducibility to analyze phytoestrogens.

3.2. Quantitative analysis of phytoestrogens in medicinal herbs

Most of the isoflavonoids and lignans were detected, but the mycoestrogens, which are formed from fungi, belong to the *Fusarium garminearumgenus*, and they have been identified in grain-based food [21], were not detected in every medicinal herbs. The total concentration (μg/g) of isoflavonoids were found to be very high in *Puerariae radix* and in descending order of concentration percentage, followed by *Schizandrae fructus, Sophorae radix, Atractylodis macrocephalae, Epimedii herba, Scutellariae radix, Paeoniae radix lba, Salviae miltiorrhizae, Cervi cornu pantotrichum* (from New Zealand), *Bupleuri radix, Gardeniae fructus, Cimicifugae rhizoma, Cervi cornu pantotrichum* (from Soviet Russia). *Ponciri fructus, Angelicae gigantis radix, Clematidis radix, Acanthopanacis, Cordyceps, Anemarrhenae rhizoma, Magnoliae cortex, Asari radix,* and *Angelicae dahuricae radix.*

Puerariae radix (Leguminosae family) contained the largest amount of genistein, biochanin-A, and daidzein among the tested herbs [22].

We detected lignans such as enterodiol and enterolactone in most of the herbs at low concentrations.

Table 3
Day-to-day variations of phytoestrogens as TMS derivatives[a]

Compound					
Flavone					
Concentration added (μg/g)	100	200	500	1000	2000
Concentration founded (Mean ± SD, μg/g)	107.34 ± 7.70	200.69 ± 2.82	500.77 ± 4.21	997.84 ± 11.09	2008.09 ± 17.04
CV %	7.18	1.41	0.84	1.11	0.85
Chrysin					
Concentration added (μg/g)	4	20	50	120	200
Concentration founded (Mean ± SD, μg/g)	3.62 ± 0.18	21.77 ± 1.80	49.11 ± 1.65	117.53 ± 2.66	204.54 ± 0.46
CV %	5.00	8.26	3.36	2.26	0.23
Apigenin					
Concentration added (μg/g)	10	20	30	50	120
Concentration founded (Mean ± SD, μg/g)	9.90 ± 1.56	20.43 ± 0.33	30.13 ± 1.81	52.76 ± 2.81	121.19 ± 1.41
CV %	15.77	1.60	6.01	5.33	1.16
Kaempferol					
Concentration added (μg/g)	20	40	200	300	
Concentration founded (Mean ± SD, μg/g)	20.36 ± 1.88	39.04 ± 0.54	202.74 ± 1.16	300.67 ± 5.02	
CV %	9.25	1.37	0.57	1.67	
Quercetin					
Concentration added (μg/g)	4	20	50	100	200
Concentration founded (Mean ± SD, μg/g)	4.55 ± 0.35	21.51 ± 1.34	48.55 ± 2.94	94.94 ± 5.11	204.50 ± 2.04
CV %	7.71	6.25	6.05	5.38	1.00
Daidzein					
Concentration added (μg/g)	40	200	400	1200	2400
Concentration founded (Mean ± SD, μg/g)	44.01 ± 32.22	218.06 ± 15.96	383.52 ± 7.62	1193.89 ± 9.11	2392.85 ± 20.97
CV %	5.05	7.32	1.99	0.76	0.88
Genistein					
Concentration added (μg/g)	40	80	200	400	800
Concentration founded (Mean ± SD, μg/g)	39.28 ± 4.79	77.42 ± 5.47	203.73 ± 4.23	395.02 ± 2.53	795.62 ± 5.50
CV %	12.18	7.70	2.08	0.64	0.69
Biochanin-A					
Concentration added (μg/g)	40	120	200	400	800
Concentration founded (Mean ± SD, μg/g)	41.66 ± 4.49	119.14 ± 6.32	199.57 ± 4.47	399.20 ± 4.85	806.16 ± 8.00
CV %	10.77	5.31	2.24	1.21	0.99
Enterolactone					
Concentration added (μg/g)	5	28	50	120	300
Concentration founded (Mean ± SD, μg/g)	5.61 ± 0.66	27.81 ± 0.48	49.11 ± 1.65	117.53 ± 2.66	300.99 ± 2.14
CV %	11.70	1.72	3.36	2.26	0.71
Enterodiol					
Concentration added (μg/g)	3	5	10	20	40
Concentration founded (Mean ± SD, μg/g)	2.95 ± 0.12	5.04 ± 0.09	9.87 ± 0.27	20.05 ± 0.49	40.09 ± 0.14
CV %	4.08	1.80	2.79	2.42	0.35
Catechin					
Concentration added (μg/g)	4	20	50	160	
Concentration founded (Mean ± SD, μg/g)	3.97 ± 0.12	18.92 ± 0.54	48.11 ± 1.08	161.94 ± 0.37	
CV %	3.05	2.83	2.24	0.23	
Equol					
Concentration added (μg/g)	1	5	10	15	20
Concentration founded (Mean ± SD, μg/g)	1.03 ± 0.07	4.91 ± 0.17	10.13 ± 0.28	14.97 ± 0.18	19.92 ± 0.22
CV %	6.38	3.43	2.79	1.17	1.10

Continued

Table 3
Continued

Compound					
α-Zearalanol					
Concentration added (μg/g)	2	4	10	20	30
Concentration founded (Mean ± SD, μg/g)	2.07 ± 0.13	4.01 ± 0.04	9.93 ± 0.05	19.67 ± 0.52	29.88 ± 0.45
CV %	6.06	1.02	0.54	2.67	1.49
β-Zearalanol					
Concentration added (μg/g)	2	4	10	20	30
Concentration founded (Mean ± SD, μg/g)	2.01 ± 0.20	4.00 ± 0.04	10.01 ± 0.18	19.77 ± 0.30	30.04 ± 0.68
CV %	9.86	1.11	1.75	1.51	2.26
α-Zearalenol					
Concentration added (μg/g)	2	4	10	20	30
Concentration founded (Mean ± SD, μg/g)	1.80 ± 0.16	4.11 ± 0.39	10.13 ± 0.11	20.00 ± 0.28	30.16 ± 0.34
CV %	9.01	9.40	1.06	1.38	1.13
β-Zearalenol					
Concentration added (μg/g)	2	4	10	20	30
Concentration founded (Mean ± SD, μg/g)	1.77 ± 0.06	4.30 ± 0.43	10.12 ± 0.20	19.91 ± 0.24	30.06 ± 0.38
CV %	2.41	10.02	2.00	1.22	1.25
Zearalenone					
Concentration added (μg/g)	2	4	10	20	30
Concentration founded (Mean ± SD, μg/g)	2.18 ± 0.36	3.96 ± 0.40	9.83 ± 0.15	19.77 ± 0.30	29.95 ± 0.51
CV %	16.61	10.14	1.52	1.51	1.71

4. Discussion

HPLC determination has been used to separate and quantify phytoestrogens such as isoflavonoids and lignans in plants or food. However, it did not have sufficient sensitivity and specificity for plants or food at a low concentration of the phytoestrogens [22]. Recently, GC-MS determination has appeared to be the method of choice to assay for phytoestrogens [20,27]. It has higher sensitivity and selectivity to determine phytoestrogens than HPLC analysis. However, suitable hydrolysis and extraction procedures were essential to detect the free form of phytoestrogens, as well as the glycoside conjugate of phytoestrogens. Mazur et al. [20] used both of enzymatic and acidic hydrolysis to get the free phytoestrogens from their glycosides. The glycoside of isoflavonoids was hydrolyzed during the enzymatic hydrolysis with β-glucuronidase/aryl sulfatase, but only a small amount of lignan glycosides was hydrolyzed in this step. Thus, lignan glycosides require more severe hydrolysis conditions such as acidic hydrolysis to produce the corresponding free lignans. However, enzyme hydrolysis must be undergone before acidic hydrolysis, because the isoflavonoids are very sensitive and break down easily in the acidic condition. The recoveries through those hydrolysis procedures were more than 90% in every phytoestrogen in our preliminary study (data not shown). Therefore, we performed the same hydrolysis procedure used by Mazur et al. for all of our analyses.

After the hydrolysis and extraction, Mazur et al. performed two kinds of anion-exchange chromatography to get rid of impurities such as neutral steroids, organic acid, and considerable amounts of chromogens [20]. However, those chromatography steps demand several careful and elaborate treatments for the preconditioning of the sephadex resin and for sample-loading and organic solvent elution. Moreover, those processes were so dependent on individual experimental skill that they led to significant losses of the compounds even though detailed experiments were performed.

For these reasons, we tried to improve the column-chromatography steps with a more convenient and less sensitive resin. We introduced Oasis HLB extraction cartridge (Waters Co., Milford, MA) instead of sephadex resin. This was more convenient to precondition and treat, and it was less pH dependent and less sensitive to the polarity of compounds than was sephadex resin. This method also made it possible to reduce complex two-stage column chromatography steps to a simple one-step column chromatography step.

Normally, after the hydrolysis and extraction, we performed derivatization with suitable derivatizing agents to decrease the polarity of the compounds and to increase the sensitivity of the compounds in GC-MS. In previous reports, hexamethyldisilane and bis(trimethylsilyl)trifluoroacetatamide containing 1% TMCS in pyrimidine were used for derivatizing agents [14,20,23,24]. Those derivatization agents could form O-TMS functional groups from the reaction with hydroxyl groups in the phytoestrogens. However, we used a mixture of MSTFA, NH_4I, and DTE (the ratio

Table 4
Concentration (μg/500g of dried herb) of phytoestrogen contents in each traditional medicine herb

Botanical Name	Fla	Chr	Api	Kae	Que	Dai	Gen	Bio	Enl	Ent	Cat	Equ
Umbelliferaes family												
Bupleuri Radix	nd	88.3	67.6	2040.2	2164.5	nd	nd	nd	nd	nd	nd	nd
Angelicae dahuricae radix	nd	nd	13.2	25.8	28.3	nd	nd	nd	nd	2.0	nd	nd
Angelicae gigantis radix	nd	nd	55.0	107.3	92.8	nd	495.2	36.3	nd	11.2	nd	0.1
Ranunculaceaes family												
Clematidis radix	nd	28.3	51.9	161.4	445.0	nd	nd	nd	18.5	nd	nd	nd
Cimicifugae rhizoma	1585	21.4	61.7	1058.3	410.0	nd	1056.2	nd	22.3	6.0	33.9	nd
Paeoniae radix lba	nd	17.3	60.3	1135.2	10401.9	996.2	580.7	369.3	40.2	nd	4420.1	nd
Magnoliaceaes family												
Magnoliae cortex	nd	17.0	18.1	40.1	106.3	nd	nd	nd	5.7	1.6	nd	nd
Schizandrae fructus	nd	nd	70.3	243.8	2244.1	152273.7	78705.4	31002.5	nd	nd	nd	nd
Leguminosaes family												
Sophorae radix	nd	nd	45.6	189.8	28.3	2904.5	109161.1	13303.3	nd	nd	nd	6.5
Puerariae radix	nd	nd	193.4	356.3	305.5	470703.1	978580.2	462093.2	248.0	nd	nd	10.4
Labiataes family												
Salviae miltiorrhizae	nd	nd	31.5	74.9	68.4	238.3	7678.1	684.8	nd	nd	nd	nd
Scutellariae radix	nd	1440.2	65.1	1278.1	2865.3	8011.6	nd	nd	nd	40.2	nd	nd
Haemodoraceaes family												
Anemarrhenae rhizoma	nd	nd	29.4	109.1	96.2	nd	nd	nd	nd	nd	nd	nd
Aristolochiaceaes family												
Asari radix	nd	nd	22.1	60.4	51.2	nd	nd	nd	8.9	6.5	nd	0.9
Clavicipitaceaes family												
Cordyceps	nd	17.5	nd	18.4	19.5	199.2	155.9	nd	nd	4.6	nd	nd
Rutaceaes family												
Ponciri fructus	nd	nd	840.7	20.0	28.2	nd	nd	nd	nd	nd	nd	20.7
Berberidaceaes family												
Epimedii herba	nd	nd	86.9	6969.3	13476.5	nd	nd	nd	nd	11.5	798.1	1.1
Rubiaceaes family												
Gardeniae frucrus	711.5	159.8	40.5	545.1	2796.5	nd	nd	nd	nd	nd	467.5	nd
Araliaceaes family												
Acanthopanacis	314.3	17.6	16.5	33.8	77.1	nd	nd	nd	nd	nd	26.3	nd
Compositaes family												
Atractylodis macrocephalae	nd	nd	52.3	113.5	368.0	43974.7	8726.7	7255.2	nd	nd	nd	nd
Cervidaes family												
Cervi pantotrichum cornu (From New Zealand)	nd	20.7	14.3	1165.7	190.5	2269.9	1165.7	nd	nd	16.7	nd	nd
Cervi pantotrichum cornu (From Soviet)	nd	18.2	15.8	86.5	113.5	1256.7	791.7	nd	nd	10.2	nd	nd

Calibration range overed herbs were 1/50 diluted again GC-MSD inject quantitative calculated.
Fla = Flavone; Chr = Chrysin; Api = Apigenin; Kae = Kaempferol; Que = Quercetin; Dai = Daidzein; Gen = Genistein; Bio = Biochanin-A; Enl = Enterolactone; Ent = Enterodiol; Cat = Catechin; Equ = Equol; nd = not detected.
Mycoestrogens: α- or β-zearalanol, α- or β-Zearalenol, zearalanone were not detected.

was 1000:4:5, v/w/w) to form TMS derivatives to enol-keto groups as well as hydroxyl groups in phytoestrogens [25,26]. Those TMS derivatives were more lipophilic than were derivatives reported in the previous report. Thus, we could get much better resolution and lower detection limits (0.02–0.4 μg/100 g) than in previous reports (2–3 μg/100 g) [20].

After the quantitative analysis of phytoestrogens in medicinal herbs, we found that kaempferol, quercetin, and apigenin were abundant phytoestrogens in most of medicinal herbs. It was also shown that there were largest amount of genistein and daidzein in *Puerariae radix*. Genistein and daidzein were reported to be the most biologically active phytoestrogens [28,29].

Therefore, our results indicate that this assay method is helpful, reproducible, sensitive and reliable in evaluating phytoestrogens. We believe that this method is readily applicable to many areas of nutritional and medicinal science.

References

[1] Davis SR, Murkies AL, Wiley G. Phytoestrogens in clinical practice. Integrative Med 1998;1:27–34.
[2] Dai Q, Franke AA, Jin F, Shu XO, Hebert JR, Custer LJ, Cheng J, Gao YT, Zheng W. Urinary excretion of phytoestrogens and risk of breast cancer among Chinese women in Shanghai. Cancer Epidemiol Biomarkers Prev 2002;11:815–21.
[3] Weber KS, Setchell KD, Stocco DM, Lephart ED. Dietary soy-phytoestrogens decrease testosterone levels and prostate weight without altering LH, prostate 5α-reductase or testicular steroidogenic

acute regulatory peptide levels in adult male Sprague-Dawley rats. J Endocrinol 2001;170:591–9.
[4] Rose DP, Boyar AP, Wynder EL. International comparison of mortality rates for cancer of the breast, ovary, prostate and colon and percapita food consumption. Cancer 1986;58:2363–71.
[5] Aldercreytz H. Western diet and western diseases; some hormonal and biochemical mechanisms and association. Scand J Clin Lab Invest 1990;50:3–23.
[6] Agnusdei D, Adami S, Cervetti R, Crepaldi G, Dimunno O. Effects of ipriflavone on bone mass and calcium metabolism in postmenopausal osteoporosis. Bone Miner 1992;19:43–8.
[7] Arjmandi BH, Alekel L, Hollis BW, Amin D, Stacewicz-Sapuntzakis M. Dietary soybean protein prevents bone loss in an ovariectomized rat of osteoporosis. J Nutr 1996;126:161–7.
[8] Pan Y, Anthony M, Watson S, Clarkson TB. Soy phytoestrogens improve radical arm maze performance in ovariectomized retired breeder rats and do not attenuate benefits of 17β-estradiol treatment. Menopause 2000;7:230–5.
[9] Howes MJ, Perry NS, Houghton PJ. Plants with traditional uses and activities, relevant to the management of Alzheimer's disease and other cognitive disorders. Phytother Res 2003;17:1–18.
[10] Anthony MS, Clarkson TB, Weedle DL. Effect of soy protein phytoestrogens on cardiovascular risk factors in rhesus monkeys. J Nutr 1995;125:803–7.
[11] Sirtori CR, Lovati MR. Soy proteins and cardiovascular disease. Curr Atheroscler Rep 2001;3:47–53.
[12] Mizutani K, Ikeda K, Kawai Y, Yamori Y. Protective effect of resveratrol on oxidative damage in male and female stroke-prone spontaneously hypertensive rats. Clin Exp Pharmacol Physiol 2001; 28:55–9.
[13] Timothy JO, Janice FW, Carl LK. Chronic consumption of flavanol- and procyanindin-rich diet is associated with reduced levels of 8-hydroxy-2′-deoxyguanosine in rat testes. J Nutr Biochem 2003;14:104–10.
[14] Mazur W, Fotsis T, Wahala K, Ojala S, Salakka A, Adlercreutz H. Isotope dilution gas chromatographic-mass spectrometric method for the determination of isoflavonoids, coumestrol, and lignans in food samples. Anal Biochem 1996;233:169–80.
[15] Murphy PA, Song T, Buseman G, Barua K. Isoflavones in soy-based infant formulas. J Agric Food Chem 1997;45:4635–8.
[16] Franke AA, Hankin JH, Yu MC, Maskarinec G, Low SH, Custer LJ. Isoflavone levels in soy foods consumed by multiethnic in populations in Singapore and Hawaii. J Agric Food Chem 1997;45:977–86.
[17] Bluck LJC, Jones KS, Thomas J, Liggins J, Harding M, Bingham SA, Coward WA. Quantitative analysis using gas chromatography/combustion/isotope ratio mass spectrometry and standard addition of intrinsically labeled standards (SAIL) application to isoflavones in foods. Rapid Commun Mass Spectrom 2002;16:2249–54.
[18] Gu X, Chu Q, O'Dwyer M, Zeece M. Analysis of resveratrol in red wine by capillary electrophoresis. J Chromatogr A 2000;881:471–81.
[19] Lee SH, Choi SY, Kim H, Hwang JS, Lee BG, Gao JJ, Kim SY. Mulberroside F isolated from the leaves of *Morus alba* inhibits melanin biosynthesis. Biol Pharm Bull 2002;25:1045–8.
[20] Mazur W, Fotsis T, Wahala K, Ojala S, Salakka A, Adlercreutz H. Isotope dilution gas chromatographic-mass spectrometric method for the determination of isoflavonoids, coumestrol, and lignans in food samples. Anal Biochem 1996;233:169–80.
[21] Mitterbauer R, Weindorfer H, Safaie N, Krska R, Lemmens M, Ruckenbauer P, Kuchler K, Adam G. A sensitive inexpensive yeast bioassay for the mycotoxin zearalenone and other compounds with estrogenic activity. Appl Environ Microbiol 2003;69:805–11.
[22] Wang X, Wu J, Chiba H, Umegaki K, Yamada K, Ishimi Y. Puerariae radix prevents bone loss in ovariectomized mice. J Bone Miner Metab 2003;21:268–75.
[23] Liggins J, Grimwood R, Bingham SA. Extraction and quantification of lignan phytoestrogens in food and human samples. Anal Biochem 2000;287:102–9.
[24] Tekel J, Daeseleire E, Heeremans A, van Peteghem C. Development of a simple method for the determination of Genistein, Daidzein, Biochanin-A, and formononetin (Biochanin-B) in human urine. J Agric Food Chem 1999;47:3489–94.
[25] Choi MH, Kim KR, Hong JK, Park SJ, Chung BC. Determination of non-steroidal estrogens in breast milk, plasma, urine and hair by gas chromatography/mass spectrometry. Rapid Commun Mass Spectrom 2002;16:2221–8.
[26] Yang YJ, Lee SH, Chung BC. Simultaneous determination of urinary phytoestrogens and estrogens by gas chromatography-mass spectrometry. Anal Sci Technol 1998;11:5–9.
[27] Mazur WM, Duke JA, Wahala K, Sirpa R, Adlercreutz H. Isoflavonoids and Lignans in legumes: nutritional and health aspects in humans. Nutr Biochem 1998;9:193–200.
[28] Kulling SE, Lehmann L, Metzler M. Oxidative metabolism and genotoxic potential of major isoflavone phytoestrogen. J Chromatogr B Anal Technol Biomed Life Sci 2002;777:211–8.
[29] Boue SM, Wiese TE, Nehls S, Burow ME, Elliott S, Carter-Wientjes CH, Shih BY, McLachlan JA, Cleveland TE. Evaluation of the estrogenic effects of legume extracts containing phytoestrogens. J Agric Food Chem 2003;51:2193–9.