GC-MS Analysis of Various Phytoestrogens in Health Functional Foods

Yu Ra Kim,[†,‡] Hee Soo Pyo,[†] Bong Chul Chung,[†] Myeong Hee Moon,[‡] and Jeongae Lee[†,*]

[†]*Molecular Recognition Research Center, Korea Institute of Science and Technology, Seoul 136-791, Korea. *E-mail: frans@kist.re.kr*
[‡]*Department of Chemistry, Yonsei University, Seoul 120-749, Korea*
Received December 26, 2016, Accepted February 10, 2017, Published online March 22, 2017

This study aimed to measure phytoestrogen contents in health functional foods (HFFs) to alleviate menopausal symptoms that are commercially available in South Korea. A sensitive and selective method using gas chromatography–mass spectrometry (GC-MS) for the monitoring of 21 phytoestrogens was developed and fully validated. Phytoestrogens were extracted by liquid–liquid extraction using methyl *tert*-butylether as the solvent. Food samples were enzymatically hydrolyzed with β-glucuronidase/arylsulfatase. Before GC-MS, the analytes were derivatized to their trimethylsilyl derivatives by incubation with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane for 30 min at 80°C. The calibration curves showed good linearity ($r^2 > 0.99$) in the range of interest for each analyte. The limits of detection and quantification of the 21 phytoestrogens were in the ranges of 0.5–20 and 1–50 ng/mL, respectively. The intra- and inter-day precisions were all below 15.7%. These multivariate procedures were efficient in determining the optimal conditions using peak heights as responses.

Keywords: Phytoestrogen, Health functional food, Simultaneous, Gas chromatography–mass spectrometry

Introduction

Phytoestrogens are naturally occurring non-steroidal plant compounds that are found in various plants such as parsley, peppers, soybean, and black tea, and in the woody portions of fruits and vegetables.[1–4] The structural similarities of phytoestrogens with endogenous estrogen (17β-estradiol)[5] allow them to exert health effects by binding to the estrogen receptor, thus hindering estrogen binding. Whether they act as an agonist or antagonist relies on the endogenous estrogen level in the human endocrine system.[6,7] Phytoestrogens largely consist of polyphenols having a 15-carbon skeleton structure, which comprises two phenyl rings (A and B) and a heterocyclic ring (C) (Figure 1).[8–10] Phytoestrogens can be categorized into flavones, isoflavones, flavonols, flavans, isoflavans, lignans, and coumestans, according to substitutions including hydroxyl and methoxy groups (Table 1).[11] The configuration and total number of these substituents are the main determinants of antioxidant activity.[9]

Recently, the use of health functional foods (HFFs) containing phytoestrogens, mainly by menopausal women having decreased estrogen levels, is increasing. The health effects of phytoestrogens depend on the amounts consumed. Excessive phytoestrogen consumption may lead to adverse health effects such as fluid circulation disorder, obesity, and even cardiovascular disease.[12–14] In accordance with the intake, fully understanding either the potential risks or the benefits from consuming HFFs is essential.

Several methods have been reported for the determination of phytoestrogens in food sources, including ultraviolet–visible detectors, particularly in diodarray configuration, fluorescence detectors, electrochemical detectors, and—the most widely utilized—high-performance liquid chromatography (HPLC).[15,16] Wielogorska *et al.* (2011) and Antignac *et al.* (2009) developed a HPLC procedure for isoflavones mainly.[17–19]

Nowadays, there is an increasing interest in the application of advanced analytical techniques based on liquid chromatography (LC) or gas chromatography (GC) with mass spectrometry (MS). However, the major drawback of LC-MS is the matrix effect. An enhancement of target-compound responses because of co-eluted matrix components altering ionization efficiency has been reported.[20]

On the other hand, GC-MS has been increasingly used for the determination of phytoestrogens owing to improved specificity and sensitivity.[21–25] Kuo *et al.* (2004), Ribeiro *et al.* (2015), and Magiera *et al.* (2011) developed GC methods involving derivatization in the sample preparation stage to form trimethylsilyl (TMS) derivatives that increase volatility and improve thermal stability.[26–29] These papers reported the quantitative determination of isoflavones only. Other studies have investigated subgroups of metabolites, such as the isoflavones daidzein and genistein, which exhibit the biological activities of phytoestrogens. However, various phytoestrogens show metabolic activities through complex enzymatic mechanisms occurring in the

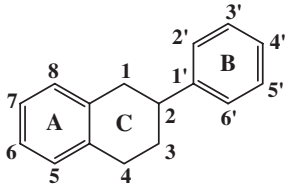


Figure 1. Basic chemical structure of phytoestrogens.

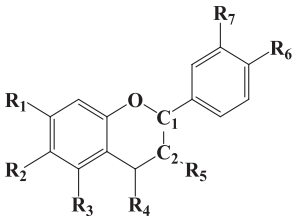
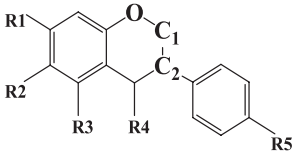
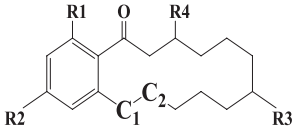
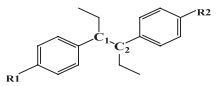
human liver, intestine, and colon.[1] Therefore, it is essential to clearly understand the effects of related phytoestrogen metabolites on human health. Thus, there is a pressing need for a standardized method that allows the simultaneous analysis of a wide range of phytoestrogens.

In this study, we developed a reliable and reproducible method for the simultaneous determination of diverse phytoestrogens in HFFs. Compared with LC-based methods, this method provided high resolution and high throughput, and had a low detection limit for all phytoestrogens. The sample preparation procedures, including enzymatic hydrolysis, liquid–liquid extraction (LLE), and derivatization, were optimized. Then, this method was applied to HFFs to alleviate menopausal symptoms, available on the Korean market.

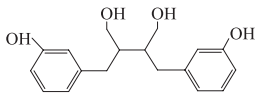
Experimental

Chemicals and Reagents. All chemicals, reagents, and solvents were of analytical grade or higher purity. Chrysin (CHR), biochanin A (BIO), daidzein (DAI), genistein (GEN), formononetin (FOR), kaempferol (KAE), quercetin (QUE), 6-methoxyflavonol (6MF), 7-methoxyflavonol (7MF), catechin (CAT), equol (EQU), enterodiol (EDO), enterolactone (ELT), α-zearalanol (α-ZLA), β-zearalanol (β-ZLA), zearalenone (ZEN), α-zearalenol (α-ZLE), β-zearalenol (β-ZLE), coumestrol (COU), hexestrol (HEX), and diethylstilbestrol (DES) were obtained from Sigma-AldrichCo. (St. Louis, MO, USA). The stable-isotope labeled internal standards (ISTD) daidzein-2,3,5,6-d_4(4-hydroxyphenyl-2,3,5,6-d_4), genistein-2,3,5,6-d_4(4-hydroxyphenyl-2,3,5,6-d_4) and (±)-catechin-2,3,4-$^{13}C_3$ were purchased from C/D/N Isotopes (Pointe-Claire, Canada) and Sigma-AldrichCo. L-Ascorbic acid, triethylamine (TEA), α-glucuronidase/arylsulfatase from *Helix pomatia*, potassium hydroxide, and derivatization reagents: *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS), *N*-methyl-*N*-(trimethylsilyl)

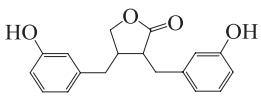
Table 1. Structures of phytoestrogens analyzed in this study

Structure	Compounds		C1–C2	R1	R2	R3	R4	R5	R6	R7
	Flavones									
	Chrysin	CHR	=	OH		OH	O			
	Kaempferol	KAE	=	OH		OH	O	OH		OH
	Quercetin	QUE	=	OH		OH	O	OH	OH	OH
	6-Methoxyflavonol	6MF	=		OCH_3		O	OH		
	7-Methoxyflavonol	7MF	=	OCH_3			O	OH		
	Catechin	CAT	–	OH	OH	OH		OH		OH
	Isoflavones									
	Biochanin A	BIO	=	OH	OH	OCH_3	O			
	Daidzein	DAI	=	OH			O	OH		
	Genistein	GEN	=	OH		OH	O	OH		
	Formononetin	FOR	=	OH	OH	OCH_3	O			
	Equol	EQU	–	OH	OH					
	Mycoestrogens									
	α-Zearalanol	α-ZLA	–	OH	OH	OH	CH_3			
	β-Zearalanol	β-ZLA	–	OH	OH	OH	CH_3			
	Zearalenone	ZEN	=	OH	OH	=O	CH_3			
	α-Zearalenol	α-ZLE	=	OH	OH	OH	CH_3			
	β-Zearalenol	β-ZLE	=	OH	OH	OH	OH			
	Synthetic estrogens									
	Hexestrol	HEX	–	OH	OH					
	Diethylstilbestrol	DES	=	OH	OH					

Lignans

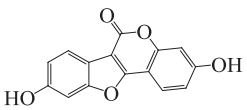


Enterodiol(EDO)



Enterolactone(ELT)

Coumestans



Coumestrol(COU)

trifluoroacetamide (MSTFA), ammonium iodide (NH_4I), and dithioerythritol (DTE) were purchased from Sigma-AldrichCo. (St. Louis, MO, USA). HPLC-grade methanol, ethanol, *tert*-butyl methyl ether (MTBE), ethyl acetate (EA), and diethyl ether (DE) were from Burdick and Jackson (Muskegon, MI, USA). Deionized water was prepared using the Milli-Q purification system (Millipore, Billerica, MA, USA).

Stock Solutions and Calibration Standards. Stock solutions of the phytoestrogen analytes were prepared in methanol at final concentration of 1000 μg/mL. For use as calibration standards, 100 μL each of stock solution was diluted in methanol in a total volume of 10 mL, and dilution series of 0.0001–10 μg/mL in methanol were prepared.

The internal standard mixture was prepared from commercially available isotopically labeled (^{2}H (D) or ^{13}C) standards: DAI-d_4, GEN-d_4, and CAT-^{13}C were mixed with methanol. One milliliter of these solutions were diluted 0.1 μg/mL with methanol (total volume of 10 mL). All standard solutions were stored in 15-mL amber glass vials in the dark at 4°C.

Sample Collection. The HFFs were collected from the market in Korea. The collected HFFs are locally used for relief of menopausal symptoms.

Sample Preparation. For the analysis of phytoestrogens, HFF samples (10 mL for liquid samples, 2 mg for powdered samples) were weighed and dissolved in 100 mL of 80% (v/v) aqueous methanol and then sonicated for 20 min. Aliquots (400 μL) of the solutions were placed in glass test tubes, and 100 μL of internal standard (100 ng/mL) was added. The mixture was evaporated to dryness under a nitrogen stream at 40°C. Before enzymatic hydrolysis, the residue was dissolved in 100 μL of L-ascorbic acid (100 mg/mL). One milliliter of 0.2 M sodium acetate buffer (pH 5.2) and 50 μL of β-glucuronidase/arylsulfatase from *H. pomatia* were added for enzymatic hydrolysis. The mixture was incubated at 55°C for 3 h to hydrolyze glycosidic forms of phytoestrogens to their aglycones. After cooling to room temperature, the mixture was extracted with 2.5 mL of MTBE by mechanical shaking for 20 min, and centrifuged at 2500 rpm for 5 min. Organic and aqueous phases were separated by freezing. The aqueous phase was re-extracted with 2.5 mL of MTBE, and extracts were combined. Fifty microliters of TEA was added in each solution, which was then evaporated to dryness under a nitrogen stream, at 40°C. The vials were placed in a vacuum desiccator over silica gel/KOH for at least 30 min, and dry residues containing phytoestrogens were reacted with BSTFA/1% TMCS by incubation at 80°C for 30 min to form TMS derivatives for direct analysis by GC-MS.

Instrumental Conditions. Phytoestrogens were analyzed on an 6890 Plus gas chromatograph coupled to a 5975 mass selective detector (MSD) equipped with an Ultra-2 (SE-54 bonded phase) fused-silica capillary column (25 m × 0.2 mm i.d., 0.33-μm film thickness) (all from Agilent Technologies, Santa Clara, CA, USA), and operated in the electron-impact ionization mode. The electron energy was 70 eV, and the ion source temperature was 230°C in selected ion monitoring modes. Each sample (2 μL) was injected in split mode (5:1) at 280°C; the oven temperature was initially at 160°C and ramped to 270°C (hold for 10 min) at 20°C/min, and finally to 300°C (hold for 4 min) at 5°C/min. Helium carrier gas was set to column head pressure of 79 kPa (column flow: 1.0 mL/min at 160°C). The MSD ChemStation software (Agilent Technologies, G1701DA, D.02.00.275) was used to control spectrometer, data acquisition, and data processing. GC peak identification—carried out on disc version—was achieved by comparing the retention times and matching the abundance ratio of three characteristic ions with those of individual standards.

Method Validation. The method was validated according to the guidelines of the US Food and Drug Administration.[30] The instrument was tuned with perfluorotributylamine by using the auto-tune program. Calibration curves were obtained to analyze blank samples spiked with standard mixture solutions of phytoestrogens. Calibration curves of the 21 phytoestrogens were used for internal quantification and were drawn at different levels, with nominal concentrations of 0.5–5000 ng/mL. Calibration controls across the concentration range were processed to obtain the linear regression parameter ($r^2 > 0.99$ for all the compounds). Calibration standards were used to assess the sensitivity and linearity of the method. Accuracy and precision of the method were tested using blank matrices (80% methanol, v/v) spiked with phytoestrogens, and the quality control samples were detected five times per day for five days.

Results and Discussion

GC-MS Analysis. Using GC for separation of phytoestrogens for simultaneous determination is particularly useful because phytoestrogens have highly similar chemical structures. Various parameters such as initial temperature, holding time, ramping rate, and carrier gas flow were optimized for all compounds. All phytoestrogens were well separated and showed symmetric peaks in the total ion chromatogram (Figure 2), permitting their identification, confirmation, and quantitation. The retention times of 24 phytoestrogens, including three internal standards, ranged from 6.3 min to 19.8 min within a 25.5-min run time.

For quantification, the characteristic ions of each phytoestrogen were determined as TMS derivatives in the selected ion monitoring mode of the electron-impact ionization. To maximize the sensitivity, the characteristic ions were monitored in six different groups. Peak identification was achieved by comparing the retention times and matching the height ratios of the characteristic ions (Table 2).

Derivatization. Because of their polar and non-volatile properties, phytoestrogens must be derivatized to make them suitable for GC-MS analysis.[31] Usually, estrogenic compounds are derivatized by TMS silylation with MSTFA

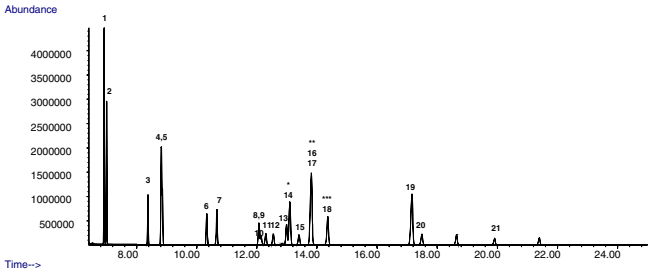


Figure 2. Total ion chromatogram for 24 phytoestrogens including three internal standards. Peak 1. HEX, 2. DES, 3. 6MF, 4. EQU, 5. 7MF, 6. EDO, 7. CHR, 8. FOR, 9. ELT, 10. α-ZLA, 11. β-ZLA, 12. ZEN, 13. BIO, 14. CAT, 15. α-ZLE, 16. DAI, 17. β-ZLE, 18. GEN, 19. COU, 20. KAE, 21. QUE. *CAT-^{13}C, **DAI-d_4, ***GEN-d_4.

or BSTFA.[32] In this study, various catalysts or additives are added to improve the silylation efficiency. As silylating reagents, MSTFA/NH_4I/DTE (1000:4:5, v/wt/wt) and BSTFA/1% TMCS (100:1, v/v) were compared (Figure 3). The reaction between MSTFA and NH_4I produces trimethyliodosilane that acts as a powerful TMS donor.[33] DTE is often added as an antioxidant to prevent addition reaction with the free iodine generated.[34] When using MSTFA/NH_4I/DTE, isoflavones yielded multiple peaks because of incomplete steric hindrance of their keto group at position 4,[22] which may mean that the silylation was not completed (Figure 3).[32] On the other hand, BSTFA is more volatile than many other silylating reagents, thereby causing less chromatographic interference. Since hydroxyl groups at the hindered position were incompletely derivatized by BSTFA alone, TMCS was added to increase the silyl donor strength.[22,35] When we used BSTFA/1% TMCS, a derivatization agent with multiple hydroxyl groups,[20] a single peak appeared and no interference was observed, and the peak intensity was higher than in case MSTFA/NH_4I/DTE was used. Therefore, BSTFA/1% TMCS was used in further experiments. Having chosen the derivatization reagents, the derivatization temperature and time were optimized (Figure 4).

These conditions play important roles in derivatization reaction yield. The effects of four reaction temperatures (40, 60, 80, and 90°C) for 30 min were evaluated. According to the results, derivatization at 80°C yielded maximum peak intensity and excellent reproducibility for all compounds (Figure 4(a)). The influence of reaction time was optimized by testing four reaction times (10, 30, 60, and 90 min) at 80°C. The result showed that peak height increased with increasing derivatization time (Figure 4(b)). However, no significant improvement in the yield was observed when reaction times were longer than 30 min (<8.3% increase in peak height from 30 to 90 min). For efficiency, a reaction time of 30 min was selected. Thus, further derivatizations were conducted with BSTFA/1% TMCS at 80°C for 30 min.

Table 2. Information on phytoestrogens as aglycones for quantitation and confirmation

Compound	M. W.	Number of TMS group	Retention time (min)	Selected ion (m/z)
Group 1				
HEX	414	2	6.8	207, 399, 177
DES	412	2	6.9	412, 397, 383
Group 2				
6MF	340	1	8.3	325, 340, 282
EQU	386	2	8.7	192, 386, 371
7MF	340	1	8.8	325, 340, 282
EDO	590	4	10.2	180, 500, 410
CHR	398	2	10.6	383, 398, 311
Group 3				
FOR	340	1	12.1	340, 325, 269
ELT	442	2	12.1	180, 442, 427
α-ZLA	610	3	12.2	433, 538, 523
β-ZLA	610	3	12.5	433, 538, 523
ZEN	534	3	12.8	519, 534, 305
Group 4				
BIO	428	2	12.9	413, 428, 370
CAT	650	5	13.0	368, 650, 560
*CAT-^{13}C	653	5	13.0	370, 653, 563
α-ZLE	536	3	13.3	305, 536, 431
*DAI-d_4	402	2	13.5	402, 387, 359
Group 5				
DAI	398	2	13.8	398, 383, 355
β-ZLE	536	3	13.8	305, 536, 431
*GEN-d_4	486	3	14.1	475, 403, 230
GEN	486	3	14.3	471, 399, 228
Group 6				
COU	430	2	17.2	412, 397, 191
KAE	574	4	17.4	559, 574, 487
QUE	662	5	19.8	647, 662, 559

* indicates internal standards; quantified ions are underlined.

Enzymatic Hydrolysis. Phytoestrogens in HFFs are present in their free form or various forms such as glucosides, etherified glucosides, and aglycones.[6] Thus, hydrolysis is essential to convert all conjugated phytoestrogens into aglycones, to analyze this single form by GC-MS. Compared with enzymatic hydrolysis, DAI, CAT, EDO, and DES were very sensitive to acidic hydrolysis, which easily broke them down. Therefore, we used enzymatic hydrolysis in further analyses. In this study, conjugated phytoestrogens were hydrolyzed with β-glucuronidase, a member of the glycosidase family, and arylsulfatase was added for hydrolyzing the sulfate forms of phytoestrogens.[17,27] Conjugated phytoestrogens were incubated with β-glucuronidase/arylsulfatase at 55°C for 3 h. For maximum enzyme activity, the hydrolysis reaction was carried out under commonly

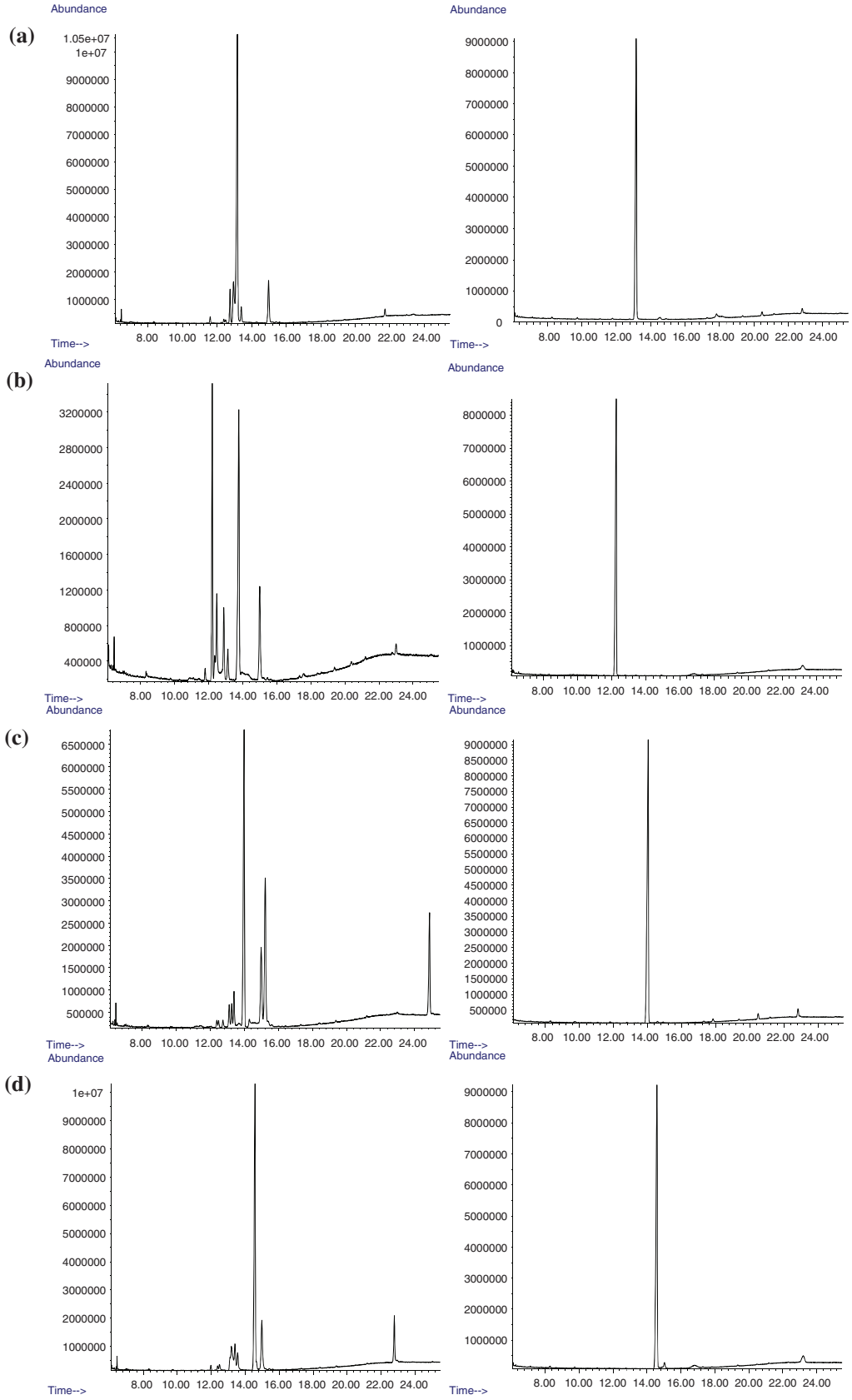


Figure 3. Comparison of derivatization reagent with MSTFA/NH_4I/DTE (left) and BSTFA/TMCS (right); (a) BIO, (b) FOR, (c) DAI, (d) FOR.

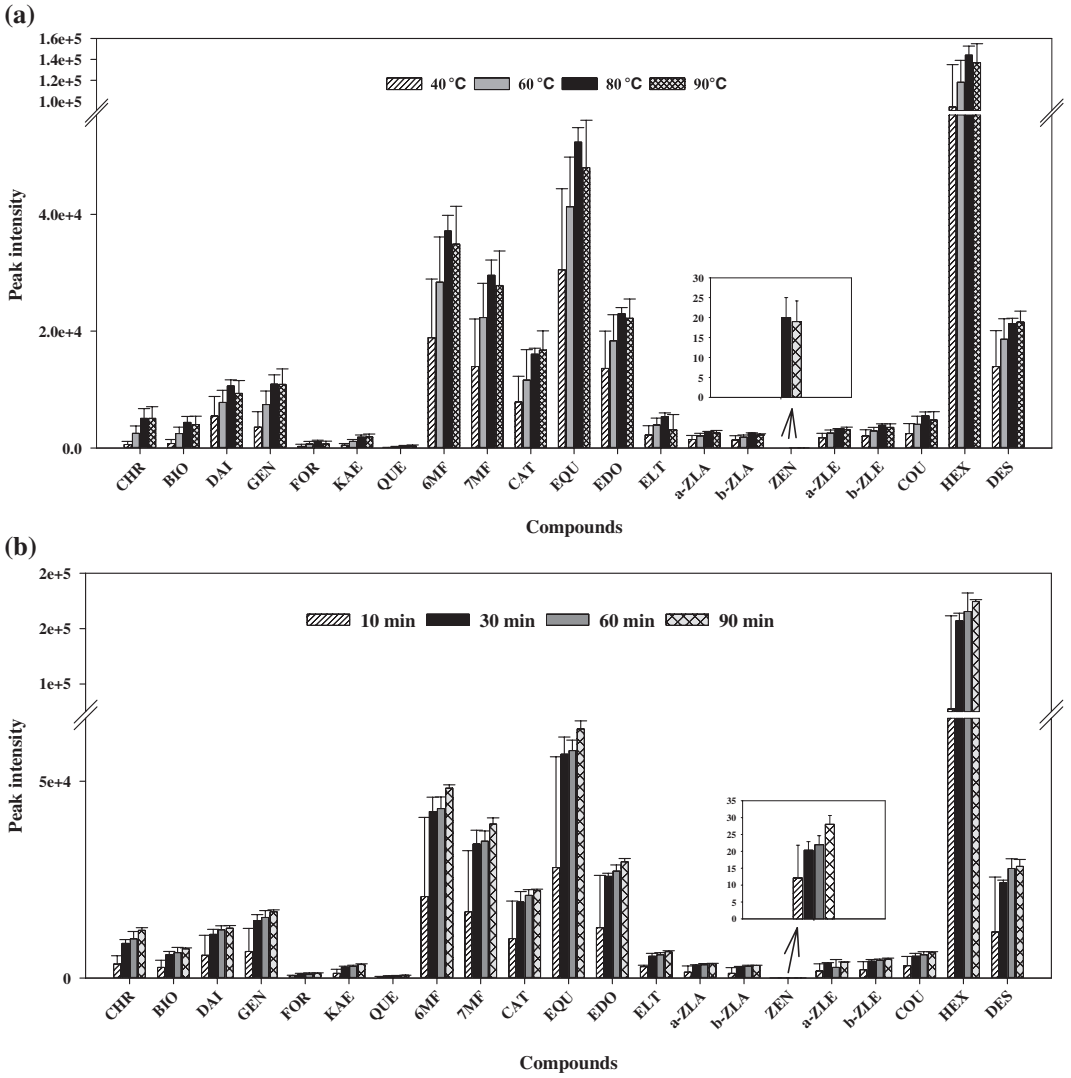


Figure 4. Optimization of derivatization conditions in GC-MS analysis.

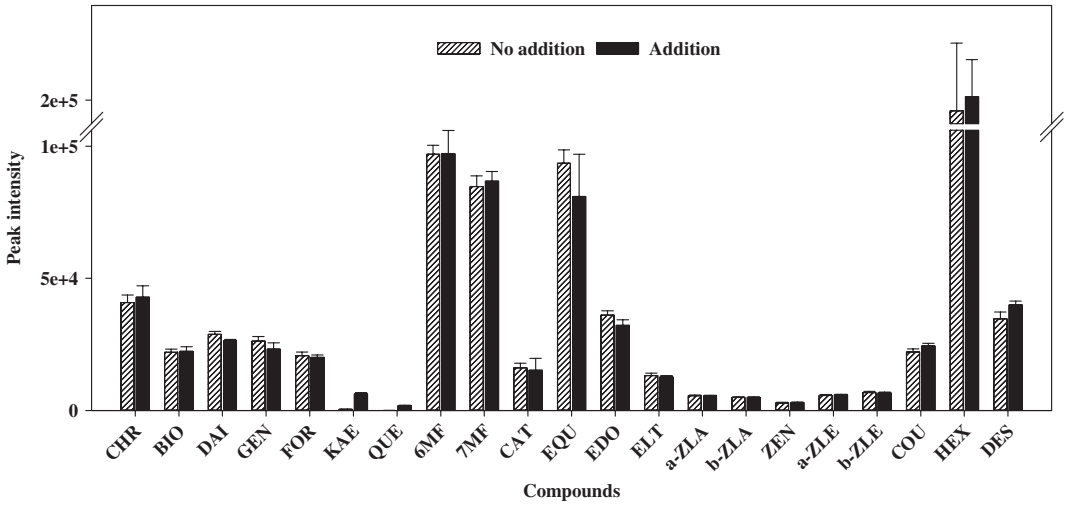


Figure 5. Effects of adding L-ascorbic acid solution before enzymatic hydrolysis.

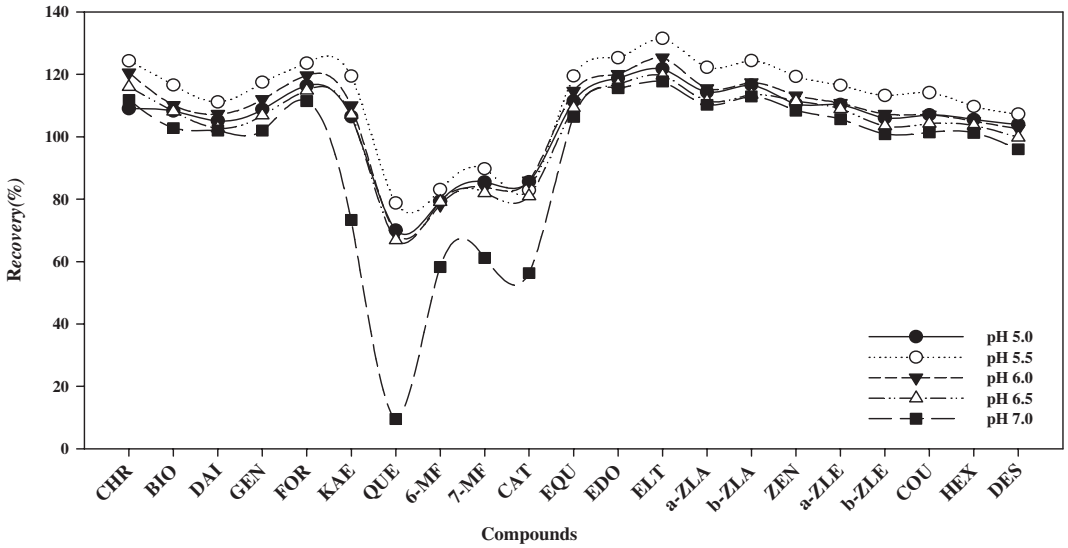


Figure 6. Effects of pH on extraction efficiency.

used condition. Phytoestrogens containing catechol groups on the B-ring undergo oxidation as the hydroxyl group at position 3 can react with other hydroxyl groups on the B-ring.[9] Because of this intramolecular hydrogen bonding, 3′,4′-catechol on the B-ring influences the hydroxyl group at position 3. This may clarify the potent antioxidant activity of this class of phytoestrogens.[9,36] Therefore, aqueous L-ascorbic acid solution was used to prevent oxidative degradation before enzymatic hydrolysis, the effects of which are shown in Figure 5. The pH for effective extraction of the components from the hydrolyzed sample was tested. At this time, pH was tested in a range of 5–7, and the best extraction efficiency was obtained at pH 5.5. On the other hand, it was confirmed that the extraction efficiency decreased with increasing pH. Thus, no further pH adjustment was performed after hydrolysis at pH 5.2. Overall, extraction recoveries ranged from 80 to 120%, and no significant decreases in daidzein and genistein were observed (Figure 6).

Extraction. After enzymatic hydrolysis, LLE was used for the selective separation of deconjugated phytoestrogens from interfering components within the matrix. LLE is useful for separating analytes from interferences by partitioning the sample between two immiscible liquids or phases. The first step in the LLE optimization procedure was to select extraction solvents. An optimum-polarity organic solvent can be conveniently selected to extract phytoestrogens from HFFs. MTBE (Polarity Index, P' 2.5), DE (P' 2.8), and EA (P' 4.4) were evaluated for their efficiency as extraction solvents having different polarity. CAT and EQU showed the lowest yield and low reproducibility above 30% of the relative standard deviation (RSD) when extracted with DE. EA, with higher P' than others, showed the lowest extraction efficiency of the three solvents. Meanwhile, MTBE showed good extraction efficiency with high reproducibility for all compounds and was therefore selected for further experiments.

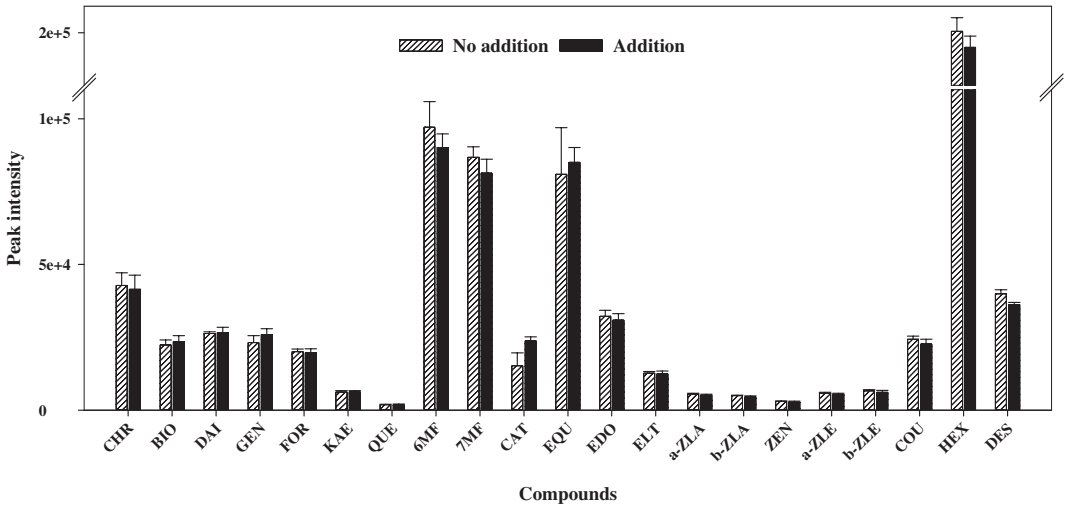


Figure 7. Effects of adding TEA before evaporation under nitrogen stream.

Table 3. Calibration curves and validation results of overall method for phytoestrogens

Compounds	Calibration curve	Linearity (r^2)	Range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	Intra-day (n = 5)		Inter-day (n = 5)		Recovery (n = 5, %)
						Precision (%CV)	Accuracy (%)	Precision (%CV)	Accuracy (%)	
CHR	$y = 65.4360x - 0.1311$	0.9992	2–200	2	5	3.8	93.4	14.7	78.4	109.1
KAE	$y = 34.3150x - 0.1446$	0.9993	10–1000	10	20	4.4	91	10.4	79	104.2
QUE	$y = 23.6970x - 0.7676$	0.9952	20–2000	20	50	3.5	129.1	9.6	129.2	89.5
6MF	$y = 132.6200x + 2.5126$	0.9976	10–1000	10	20	2.5	104.9	2.8	104.4	102.9
7MF	$y = 136.2400x + 3.1772$	0.9928	10–1000	10	20	4.3	116.2	14.1	106.8	88.8
CAT	$y = 29.3100x + 0.0146$	0.9961	0.5–50	0.5	1	7.9	105.6	11.9	110.4	105
BIO	$y = 43.1020x + 0.0062$	1.0000	0.5–50	0.5	1	5.6	97.5	9.3	101.2	101.2
DAI	$y = 53.5352x + 0.0041$	0.9996	0.5–50	0.5	1	4.1	99.8	5.5	96.3	114
GEN	$y = 40.5140x - 0.0011$	0.9998	2–200	2	5	4.4	102.8	8.8	96.3	111.8
FOR	$y = 30.4430x + 0.0435$	0.9987	1–100	1	2	4.7	103.4	2.9	100.8	83.2
EQU	$y = 74.7990x + 0.1359$	0.9985	1–100	1	2	2.8	110.8	13.5	98.5	105.5
α-ZLA	$y = 14.2840x + 0.0019$	1.0000	1–100	1	2	4.3	112.7	12.4	102.6	110.3
β-ZLA	$y = 12.1580x - 0.0014$	0.9999	1–100	1	2	5.1	114.1	9.4	104.1	98.4
ZEN	$y = 0.0423x + 0.0029$	0.9915	20–2000	20	50	2.2	102.7	6.3	105.8	106.7
α-ZLE	$y = 7.9516x - 0.0016$	0.9998	1–100	1	2	3.6	100.6	6.4	108.7	112
β-ZLE	$y = 8.296x + 0.0470$	0.9995	1–100	1	2	7.3	100.6	12.3	115.3	99.6
EDO	$y = 45.7000x + 0.0444$	0.9973	1–100	1	2	2.7	107.1	6.9	104.8	104.7
ELT	$y = 19.3520x + 0.0102$	0.9996	1–100	1	2	2.3	104.3	5.3	102.3	108.6
COU	$y = 63.5390x - 0.0213$	0.9997	0.5–50	0.5	1	2.2	106.4	4.8	103.7	87.9
HEX	$y = 324.3400x + 3.8300$	0.9989	0.5–50	0.5	1	3.6	111	15.7	97.6	118.8
DES	$y = 132.5900x + 0.0062$	0.9994	0.1–10	0.1	0.2	4.8	106.9	13.3	105.2	114.4

In the next step, various LLE extraction volumes were tested. The concentration of analytes remaining in aqueous solution decreases after several extractions with an organic solvent. Thus, it is always better to use several small portions of solvent to extract a sample than to extract with one large portion.[37] Improved efficiency of multiple extractions drops rapidly as the total volume is subdivided into smaller portions.[38] Three extraction volume regimens were tested; extraction with 2.5 or 5 mL twice, and extraction with 5 mL once. Extraction twice with 2.5 mL of solvent was substantially more effective than extraction with 5 mL once. Extraction with 5 mL twice resulted in loss of volatile compounds because of the long time needed for evaporating a large amount of combined extracts.

After extraction, TEA was added to the combined extracts as an acid neutralizer for condensation reactions and to prevent volatilization before evaporation under a nitrogen stream. During evaporation, condensation of CAT may occur by intermolecular C–C bond formation between catechol rings.[39,40] The effects of adding TEA are shown in Figure 7; overall, the extraction efficiencies of all compounds were improved.

Method Validation. The established method was validated for linearity, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy. A calibration curve was generated for all reference standards using blank matrix (80% methanol, v/v) at 16 different concentrations. Phytoestrogens in validation samples were quantified using the ratios of the peak heights of the phytoestrogen to those of the ISTDs. The proposed method was found to be linear ($r^2 > 0.99$) over the working range. The LOD and LOQ were in the range 0.1–2000 ng/mL for all phytoestrogens. Precision and accuracy were examined through analyzing validation samples at four different concentrations for each compound. Intra-day (n = 5) precision was 2.2–7.9%, while accuracy was 91.0–129.1%, and inter-day (n = 5) precision and accuracy were 2.8–15.7 and 78.4–129.2%, respectively. Recoveries were measured at the same four concentrations as precision and accuracy were obtained for each compound (n = 5). The overall recovery was 83.2–118.8% for each phytoestrogen studied (Table 3). Good recoveries were obtained for all phytoestrogens, ranging from 78.5 to 123.8% for the liquid-type and from 77.2 to 119.1% for the tablet-type HFFs. These results indicated that our sample preparation method was suitable for the analysis of phytoestrogens in various samples, despite the complexity of the different matrices.

Analysis of Phytoestrogens in HFFs. To our knowledge, this is the first report of a procedure to analyze various phytoestrogens in HFFs simultaneously. The established GC-MS-based method was successfully applied to determine phytoestrogens in HFFs. In this study, 59 types of HFFs used for improvement of menopausal syndrome, including 30 liquid-type and 29 tablet-type HFFs, were analyzed. These are complex extracts based on pomegranate, evening primrose oil, propolis, soybean extract, and various other

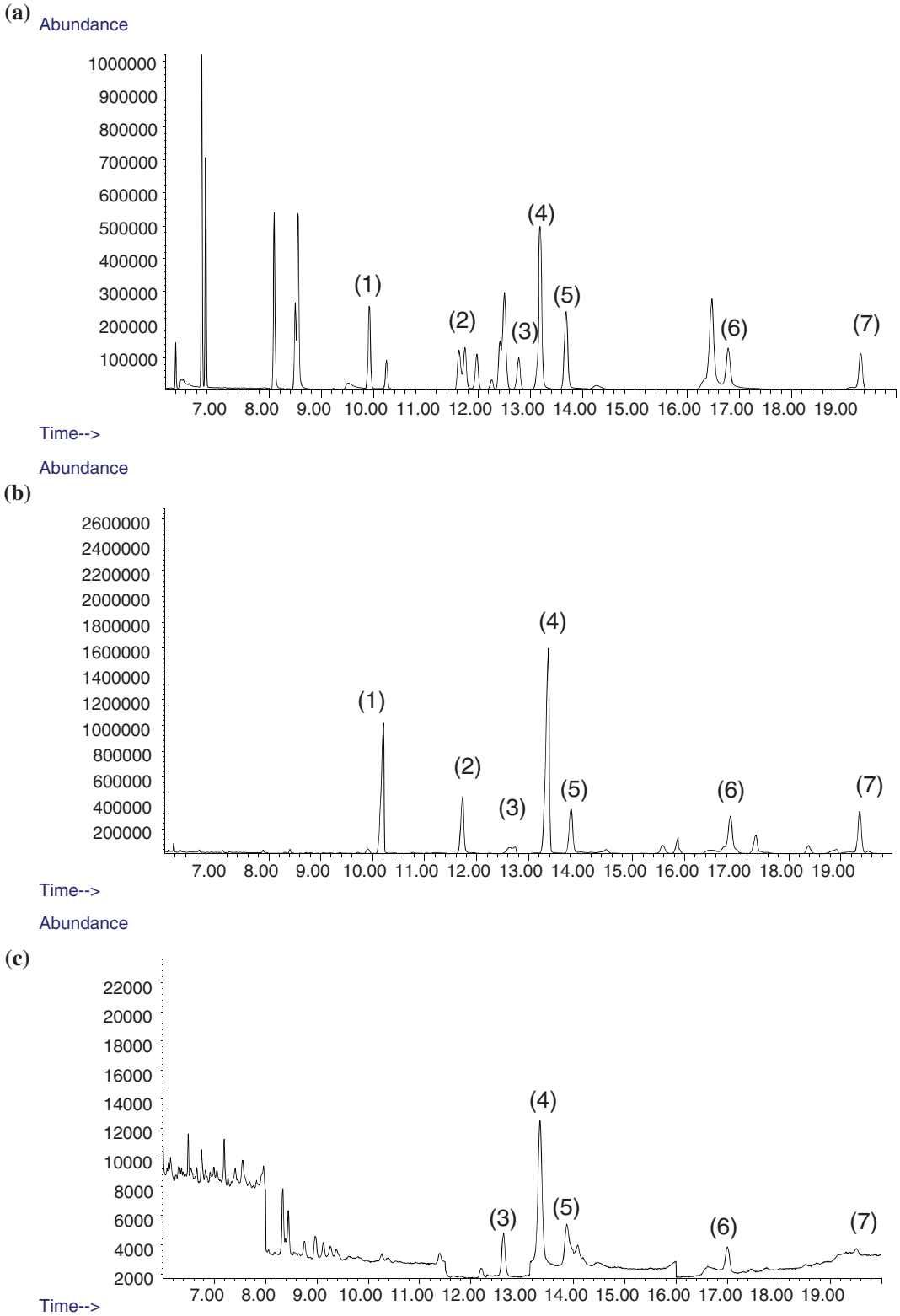


Figure 8. Total ion chromatogram of reference standard at 1 μg/mL (a), in liquid-type (b) and in tablet-type (c); Peak (1) CHR, (2) FOR, (3) CAT, (4) DAI, (5) GEN, (6) KAE, (7) QUE.

ingredients. The chromatograms of the samples are shown in Figure 8. Table 4 summarizes the results. The concentration detected in the real sample is the value to which the dilution factor is applied. Among the phytoestrogens monitored, all except 7MF and ELT were positively detected. More phytoestrogens were detected in liquids than in tablets. All of the samples contained the flavone KAE, which was present at concentrations of 0.284–1382.497 μg/mL in liquids, and of

Table 4. Determined phytoestrogen contents in HFFs

	Liquids (n = 39)			Tablets (n = 20)		
Compounds	Average (μg/mL)	Range (μg/mL)	Detection frequency (%)	Average (mg/g)	Range (mg/g)	Detection frequency (%)
CHR	1056.87	4.009–3931.824	15.4	0.933	0.933	5.0
BIO	5.326	0.014–56.305	43.6	654.600	0.15–1963.4	15.0
DAI	170.737	0.017–12.104	89.7	10.298	0.149–60.800	90.0
GEN	202.817	0.059–1026.892	43.6	236.655	1.31–2147.706	55.0
FOR	139.789	4.155–1430.794	56.4	36.022	7.069–117.212	70.0
KAE	74.501	0.284–1382.497	100.0	57.870	1.106–1117.760	100.0
QUE	2090.505	1.862–8592.495	28.2	8.799	5.947–1117.650	10.0
6MF	154.906	154.906	2.6	N.D.	–	–
7MF	N.D.	–	–	N.D.	–	–
CAT	445.546	44.785–968.368	12.8	205.796	16.000–557.429	15.0
EQU	145.857	0.041–600.868	18.0	N.D.	–	–
EDO	9.898	0.032–32.000	10.3	N.D.	–	–
ELT	2713.165	10.250–24849.876	53.9	N.D.	–	–
α-ZLA	0.893	0.023–2.456	23.1	0.244	0.082–0.369	25.0
β-ZLA	1.469	0.540–2.954	12.8	0.105	0.208	5.0
ZEN	11594.705	54.560–54343.027	12.8	34.702	34.702	5.0
α-ZLE	7.016	0.729–21.948	18.0	N.D.	–	–
β-ZLE	600.873	13.092–2895.301	12.8	10.287	10.287	5.0
COU	0.983	0.047–2.178	10.3	N.D.	–	–
HEX	2.570	0.012–5.129	5.1	N.D.	–	–
DES	0.434	0.001–1.785	84.6	3.664	0.021–0.794	75.0

N.D., analyte was not detected.

1.106–1117.760 mg/g in tablets. Intake of KAE, mainly found in fruits, broccoli, and many berries, is known to correlate with a low incidence of ovarian cancer in postmenopausal women, and KAE has been shown to sensitize cancer cells to anti-cancer agents. Hence, it is currently considered as a possible cancer therapy agent. DES, which acts as a full agonist or antagonist of all types of estrogen-related receptors, can be derived from anethole that is used as a flavoring substance. It showed high detection frequencies above 80%, although generally at very low concentrations (0.001–1.785 μg/mL for liquids, 0.021–0.794 mg/g for tablets). KAE and DES were followed by the isoflavones, BIO, DAI, GEN, and FOR, with high detection rates (>40%) in both sample types.

The nutritional information on the HFFs did not clearly show the content of each phytoestrogen component. Our analysis showed that various kinds of phytoestrogens are present in the HFFs. For the complex extracts mainly containing pomegranate and evening primrose oil, FOR showed a high detection rate, and CHR was mainly detected in the complex extracts containing propolis as a main component. Daidzein and genistein were found to be more abundant than other isoflavones in soybean extracts. Analysis of phytoestrogens has been generally focused on isoflavones such as DAI and GEN, because these have the highest biological activity and are the most widely used.[41–46] Analysis of a wide range of 21 phytoestrogen contents in HFFs has not yet been reported. Our results support that there are various types of phytoestrogens in HFFs, and that their contents vary greatly. As phytoestrogens can differ in their activities, our data may be helpful in understanding the potential effects of specific phytoestrogens found in HFFs. Further, our data should be useful in more accurately estimating the phytoestrogen intake in epidemiological and clinical studies and in designing high phytoestrogen diets in clinical trials.

Acknowledgments. This research was supported by the Bio & Medical Technology Development Program through the National Research Foundation of Korea funded by the Ministry of Science, ICT and Future Planning (NRF-2013M3A9B6046413) and by an intramural grant from the Korea Institute of Science and Technology (2E25360)

References

1. J. Yu, Q. Wu, S. Qiao, Z. Uu, N. J. B. Yu, *J. Pharm. Biomed. Anal.* **2009**, *50*, 939.
2. N. J. Turner, B. M. Thomson, I. C. Shaw, *Nutr. Rev.* **2003**, *61*, 204.
3. T. Cornwell, W. Cohick, I. Raskin, *Phytochemistry* **2004**, *65*, 995.
4. L. Adly, D. Hill, M. E. Sherman, S. R. Sturgeon, T. Fears, C. Mies, R. G. Ziegler, R. N. Hoover, C. Schairer, *Int. J. Cancer* **2006**, *119*, 2402.

5. J. I. Taylor, P. B. Grace, S. A. Bingham, *Anal. Biochem.* **2005**, *341*, 220.
6. S. Andres, U. Hansen, B. Niemann, R. Palavinskas, A. Lampen, *Food Funct.* **2015**, *6*, 2017.
7. C. Boniglia, B. Carratum, R. Gargiulo, S. Giammarioli, M. Mosca, E. Sanzini, *Food Chem.* **2009**, *115*, 1389.
8. J. A. Ross, C. M. Kasum, *Annu. Rev. Nutr.* **2002**, *22*, 19.
9. K. E. Heim, A. R. Tagliaferro, D. J. Bobilya, *J. Nutr. Biochem.* **2002**, *13*, 572.
10. J. H. Mitchell, P. T. Gardner, D. B. McPhail, P. C. Morrice, A. R. Collins, G. G. Duthie, *Arch. Biochem. Biophys.* **1998**, *360*, 142.
11. C. Fanali, A. Rocco, G. D'Orazio, L. Dugo, L. Mondello, Z. Aturki, *Electrophoresis* **2015**, *36*, 1073.
12. D. Husain, K. Khanna, S. Puri, M. Haghighizadeh, *J. Am. Coll. Nutr.* **2015**, *34*, 42.
13. J. E. Rossouw, G. L. Anderson, R. L. Prentice, *JAMA* **2002**, *288*, 321.
14. G. L. Anderson, M. Limacher, A. R. Assaf, *JAMA* **2004**, *291*, 1701.
15. G. Wang, S. S. Kuan, O. J. Francis, G. M. Ware, A. S. Carman, *J. Agric. Food Chem.* **1990**, *38*, 185.
16. B. Klejdus, J. Vacek, V. Adam, J. Zehnálek, R. Kizek, L. Trnková, V. Kubáň, *J. Chromatogr. B* **2004**, *806*, 101.
17. F. Rafii, *Metabolites* **2015**, *5*, 56.
18. E. Wielogorska, C. T. Elliott, M. Danaher, L. Connolly, *Food Chem. Toxicol.* **2014**, *69*, 260.
19. J.-P. Antignac, I. Gaudin-Hirret, H. Naegeli, R. Cariou, C. Elliott, B. Le Bizec, *Anal. Chim. Acta* **2009**, *637*, 55.
20. A. L. Capriotti, C. Cavaliere, V. Colapicchioni, S. Piovesana, R. Samperi, A. Lagana, *J. Chromatogr. A* **2013**, *1313*, 62.
21. E. Noh, C.-Y. Yoon, J. H. Lee, J. Lee, S.-Y. Baek, H. B. Oh, J.-A. Do, *Bull. Korean Chem. Soc.* **2016**, *37*, 1029.
22. H.-W. Kuo, W.-H. Ding, *J. Chromatogr. A* **2004**, *1027*, 67.
23. C. Ribeiro, M. E. Tiritan, *Int. J. Environ. Anal. Chem.* **2015**, *95*, 187.
24. S. Magiera, C. Uhlschmied, M. Rainer, C. W. Huck, I. Baranowska, G. K. Bonn, *J. Pharm. Biomed. Anal.* **2011**, *56*, 93.
25. H. Adlercreutz, T. Fotsis, C. Bannwart, K. Wähälä, G. Brunow, T. Hase, *Clin. Chim. Acta* **1991**, *199*, 263.
26. S. Moors, M. Blaszkewicz, H. M. Bolt, G. H. Degen, *Mol. Nutr. Food Res.* **2007**, *51*, 787.
27. W. Mazur, T. Fotsis, K. Wähälä, S. Ojala, A. Salakka, H. Adlercreutz, *Anal. Biochem.* **1996**, *223*, 169.
28. J. L. Peñalvo, K. M. Haajanen, N. Botting, H. Adlercreutz, *J. Agric. Food Chem.* **2005**, *53*, 9342.
29. S. C. Cunha, J. O. Fernandes, *J. Sep. Sci.* **2010**, *33*, 600.
30. Guidance for Industry: Bioanalytical Method Validation; U.S. Department of Health and Human services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center of Veterinary Medicine (CVM), September 2016. URL http://www.fda.Gov/drugs/guidancecompliaceregulationinformation/guidances/defult.Htm
31. I. Ferrer, L. B. Barber, E. M. Thurman, *J. Chromatogr. A* **2009**, *1216*, 6024.
32. W.-H. Ding, C.-C. Chiang, *Rapid Commun. Mass Spectrom.* **2003**, *17*, 56.
33. J. L. Little, *J. Chromatogr. A* **1999**, *844*, 1.
34. H. L. J. Makin, D. B. Gower, B. Kirk, *Steroid Analysis*, 2nd ed., Springer-science Business Media B.V, Dordrecht, **2009**, p. 500.
35. L. Damasceno, R. Ventura, J. Ortuno, J. Segura, *J. Mass Spectrom.* **2000**, *35*, 1285.
36. S. Kumar, A. K. Pandey, *Sci. World J.* **2013**, 2013, 16.
37. D. R. Palleros, *J. Chem. Educ.* **1995**, *72*, 319.
38. D. A. Skoog, D. M. West, F. J. Holler, S. R. Crouch, *Fundamental of Analytical Chemistry*, 8th ed., Brooks/cole, Belmont, **2000**, p. 911.
39. T. Tanaka, I. Kouno, *Food Sci. Technol. Res.* **2003**, *9*, 128.
40. E. J. Kim, D. Lee, B. C. Chung, H. Pyo, J. Lee, *Sci. Total Environ.* **2014**, *470*, 1401.
41. B. Kiss, D.-S. Popa, R. Paltinean, F. Loghin, *J. Liq. Chromatogr. Relat. Technol.* **2012**, *35*, 2735.
42. Q. Wu, M. Wang, J. E. Simon, *J. Chromatogr. B* **2004**, *812*, 325.
43. Q. Wu, M. Wang, W. J. Sciarappa, J. E. Simon, *J. Agric. Food Chem.* **2004**, *52*, 2763.
44. P. B. Grace, J. I. Taylor, N. P. Botting, T. Fryatt, M. F. Oldfield, S. A. Bingham, *Anal. Biochem.* **2003**, *315*, 114.
45. K. D. R. Setchell, L. Zimmer-Nechemias, J. Cai, J. E. Heubi, *Am. J. Clin. Nutr.* **1998**, *68*, 1453S.
46. L. U. Thompson, B. A. Boucher, Z. Liu, M. Cotterchio, N. Kreiger, *Nutr. Cancer* **2006**, *54*, 184.

