

ARTICLES

Development of a Simple Method for the Determination of Genistein, Daidzein, Biochanin A, and Formononetin (Biochanin B) in Human Urine

Jozef Tekel,[†] Els Daeseleire,[‡] An Heeremans,[‡] and Carlos van Peteghem^{*‡}

Faculty of Pharmacy, Comenius University, Odbojarov 10, SK 832 32 Bratislava, Slovak Republic, and Laboratory of Food Analysis, University of Gent, Harelbekestraat 72, B-9000 Gent, Belgium

A simple method was developed for the determination of free and/or total isoflavones daidzein, genistein, and their respective 4'-methoxy derivatives biochanin A and formononetin (biochanin B) at low levels in human urine. A solid-phase extraction on octadecyl silica (C₁₈) columns was used for the isolation of the phytoestrogens from the matrix. An extraction on a ChemElut 1010 column connected on-line to a Florisil cartridge by a Teflon stopcock was used for effective eluate purification. A mixture of dichloromethane and ethyl acetate was used for elution of the isoflavones from the columns in tandem. The isoflavones were determined as trimethylsilyl (TMS) ethers using GC/MS-SIM after separation on an HP-5MS fused silica column. TMS ethers were obtained by using BSTFA containing 1% of TMCS. For the determination of free isoflavones 6-hydroxyflavone was used as internal standard, whereas robigenin was used in the case of total isoflavone determination. Recoveries for free isoflavones under study varied from 63.5 to 89.6% at the 25 ng mL⁻¹ level and from 63.5 to 89.2% at the 5 ng mL⁻¹ level in urine. Analytical curves were linear between 5 and 25 ng mL⁻¹. Detection limits varied from 1 ng mL⁻¹ for formononetin to 2.3 ng mL⁻¹ for daidzein. Recoveries for total isoflavone determination after enzymatic hydrolysis with glucuronidase from *Helix pomatia* ranged from 56.5 to 77.1% at the 25 ng mL⁻¹ level.

Keywords: Urine; phytoestrogens; liquid/liquid extraction column; gas chromatography/mass spectrometry

INTRODUCTION

Diphenolic phytoestrogens are plant substances that show some structural similarity to estradiol-17 β and to the estradiol receptor producing estrogenic effects. There are three main groups of nonsteroidal dietary phytoestrogens, namely, the flavones, the isoflavones, and the coumestans. The main known phytoestrogens are the isoflavones genistein, daidzein, biochanin A, and formononetin, the structures of which are shown in Figure 1. Certain phytochemicals in fruits, vegetables, and grains have possible cancer-preventive properties that may inhibit tumor initiation, prevent oxidative damage, or affect steroid hormones or prostaglandin metabolism to block tumor promotion (Caragay, 1992). Most significant sources of isoflavone and coumestan phytoestrogens include soybean, soy flour, soy flakes, isolated soy protein, traditional soy food such as tofu and soy drinks, second-generation soy foods, sprouts (Reinli and Block, 1996), and other leguminous plants (Kaufman et al., 1997). The three isoflavones (genistein, daidzein, glycitein) in soybean and soybean products occur in four possible forms: the free phenolic forms,

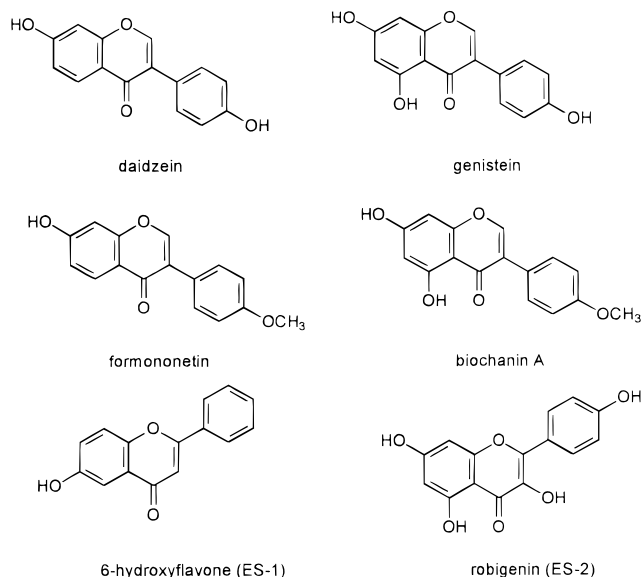


Figure 1. Chemical structures of daidzein, genistein, formononetin, biochanin A, 6-hydroxyflavone, and robigenin.

* Author to whom correspondence should be addressed (telephone 32 9 264 81 34; fax 32 9 264 81 99; e-mail carlos.vanpeteghem@rug.ac.be).

[†] Comenius University.

[‡] University of Gent.

glucosides, malonyl glucosides, and acetyl glucosides (Wang and Murphy, 1994).

Epidemiological data (Adlercreutz et al., 1991a,b, 1993a,b, 1995a,b; Franke and Custer, 1996; Lu et al.,

1995a,b) suggest that dietary legumes may be protective against breast, prostate, and colon cancer development. Populations with high isoflavone exposure through soy consumption have low cancer rates. It is well-known that the traditional Japanese diet is associated with a very low breast cancer occurrence. The low mortality from breast (Adlercreutz et al., 1991a) and prostate (Adlercreutz et al., 1993b) cancer of Japanese women and men may be due to high intake of soybean products. Isoflavonoid compounds, especially genistein and daidzein, have been implicated in cancer prevention (Adlercreutz, 1995; Messina and Barnes, 1991; Messina et al., 1994). Genistein is a specific inhibitor of tyrosine protein kinase, DNA topoisomerase II, and protein histidin kinase. All of the main isoflavonoids (genistein, daidzein), their 4'-methyl ether precursors (biochanin A, formononetin), and main isoflavonoid metabolites (equol, *O*-desmethylangolensin) detected in human and animal urine bind to the estrogen receptor (Adlercreutz et al., 1995b). Still other metabolites have been detected (Joannou et al., 1995) in human urine after soy intake. Urinary lignan and isoflavonoid excretion changed in response to alterations in vegetable, fruit, and legume intake under controlled dietary conditions (Hutchins et al., 1995; Seow et al., 1998).

Free urinary isoflavones were often analyzed by gas chromatography/mass spectrometry in single ion monitoring mode (GC/MS-SIM) after derivatization as trimethylsilyl (TMS) ethers. Different silylation agents were used, most frequently *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). Enzymatic hydrolysis of the urinary isoflavone glucuronides was carried out with β -glucuronidase/arylsulfatase from *Helix pomatia*. This procedure was used by different authors for the analysis of isoflavones and/or their metabolites in biological samples. GC/MS was used for the analysis of isoflavones in human urine (Adlercreutz et al., 1991a,b; Joannou et al., 1995; Kelly et al., 1993; Lu et al., 1995a,b), human milk (Franke and Custer, 1996), human plasma (Adlercreutz et al., 1993a,b; Morton et al., 1994), human feces (Adlercreutz et al., 1993b), beer (Rosenblum et al., 1992), and food samples (Mazur et al., 1996). An anhydrous methanolic hydrogen chloride was used for the hydrolysis (methanolysis) of steroid conjugates from male horse urine sample and was tested as an alternative (Tang and Crone, 1989).

High-performance liquid chromatography (HPLC) with a reversed phase (C_{18}) column can be directly used for the analysis of these compounds in free and conjugated form in samples without a derivatization step. This method has been used for the analysis of the isoflavone content in human urine (Franke et al., 1998), human milk (Franke and Custer, 1996), soybean foods (Wang and Murphy, 1994), legumes (Franke et al., 1994), and soybean infant formula (Murphy et al., 1997).

Investigators have used ion-exchange chromatography (Adlercreutz et al., 1991a,b, 1993a,b, 1995a; Hutchins et al., 1995; Joannou et al., 1995; Kelly et al., 1993; Mazur et al., 1996; Morton et al., 1994) or solid-phase extraction (SPE) on octadecyl silica (C_{18}) phase (Franke and Custer, 1996; Franke et al., 1998) prior to GC/MS or liquid chromatography diode array detection (LC-DAD) analysis of isoflavones in urine or plasma. The procedure developed by Lu et al. (1995a,b) for the extraction of daidzein and genistein from urine samples used liquid/liquid extraction columns. These columns were filled with diatomaceous material that absorbs and

retains water from aqueous samples. The high surface area of cartridge filling allows efficient emulsion-free interaction between the sample and the organic extraction solvent. Extraction did not require vacuum but was carried out using gravity only. Ethyl acetate was used for the elution of the isoflavones from the column. The chromatographic methods require an extensive cleanup procedure prior to analysis. Radioimmunoassay (RIA) methods are a simple alternative and are used for screening purposes. A radioimmunoassay for the analysis of formononetin in blood plasma and rumen fluid of wethers fed red clover (Wang et al., 1994) utilized antibodies raised against a formononetin-7-*O*-(carboxymethyl) ether hapten (bovine serum albumin, BSA). The tracer used was a ^3H -labeled derivative of formononetin. The radioimmunoassay for identification of daidzein and genistein in human biological fluids (serum, urine) was established on antibodies against daidzein-4'-*O*-CME-BSA and genistein-4'-*O*-CME-BSA. Both methods (Lapcik et al., 1997; Hampl et al., 1998) used ^{125}I -labeled tracer. RIA was used also for identification of the isoflavonoids in beer (Lapcik et al., 1998).

In this paper a fast, simple, and effective isolation and cleanup procedure for the determination of low isoflavone levels in human urine by GC/MS is reported.

EXPERIMENTAL PROCEDURES

Chemicals and Standards. Methanol and water of HPLC grade were obtained from BDH (Poole, Dorset, U.K.). Dichloromethane (stabilized with 20 ppm amylene) p.a. and ethyl acetate were obtained from Acros Organics (Geel, Belgium). The standards of daidzein (7,4'-dihydroxyisoflavone), genistein (5,7,4'-trihydroxyisoflavone), and biochanin A (5,7-dihydroxy-4'-methoxyisoflavone) were obtained from ICN Biomedical (Aurora, OH). The standard of formononetin (7-hydroxy-4'-methoxyisoflavone) was obtained from Extrasynthese (Genay, France). The standard of 6-hydroxyflavone (external standard 1, ES-1) was purchased from Sigma Chemical Co. (St. Louis, MO), whereas the standard of robigenin (3,5,7,4'-tetrahydroxyflavone; kaempferol) (external standard 2, ES-2) was from Fluka Chemie AG (Buchs, Switzerland). BSTFA containing 1% of trimethylchlorosilane (TMCS) was obtained from Alltech Associates, Inc. (Deerfield, IL). *Helix pomatia* juice [β -glucuronidase (5.5 units/mL) and arylsulfatase (2.6 units/mL)] were obtained from Boehringer (Mannheim, Germany).

Special indicator paper pH 4.0–7.0, acetic acid, and sodium acetate trihydrate were from Merck (Darmstadt, Germany).

Octadecyl (C_{18}) disposable extraction columns (500 mg) were from J. T. Baker (Phillipsburg, NJ). ChemElut columns CE-1010 (part 1219-8007) for liquid/liquid extraction were from Varian Sample Preparation Products (Harbor City, CA), and the Florisil Sep-Pak cartridges (part 51960) were from Millipore Corp. (Milford, MA). The extraction system Baker-10 for SPE was from J. T. Baker Chemical Co.

The derivatization vials were silylated with a solution of 5% dimethyldichlorosilane (Merck) in toluene (Merck) before use.

Urine samples were supplied by people working in the laboratory. Rubber gloves were worn when handling the urine samples.

For the determination of free isoflavones 6-hydroxyflavone (ES-1) was used as external standard, whereas robigenin (ES-2) was used for the total isoflavone determination.

Sample Cleanup Procedure. Twenty milliliters of urine sample was centrifuged for 10 min at 3600 rpm. After centrifugation, 3 mL of acetate buffer (3 mol L⁻¹, 9.9 g of acetic acid and 18.4 g of sodium acetate trihydrate in 100 mL of water) was added to the decanted supernatant of the urine. The content of the glass tube was premixed and pH was controlled using an indicator strip and adjusted if necessary with acetic acid to a value between 4.7 and 5.2. The sample

Table 1. GC Retention Times of Standards of Isoflavones and Selected Ions for the Selected Ion Monitoring

compound	t_R (min)	selection of ions (m/z)
6-hydroxyflavone (ES-1)	12:41	310, 295, 236, 165
formononetin	15:01	340, 325, 269, 226
biochanin A	15:38	413, 370, 341, 269
daidzein	16:10	398, 383, 355, 253
genistein	16:31	471, 399, 355, 327
robinetin (ES-2)	18:42	559, 487, 458, 415

was preconcentrated on a C_{18} column that had previously been conditioned with 2×5 mL of methanol and 2×5 mL of water. After application of the urine, the cartridge was washed with 2×5 mL of water and the free isoflavones were eluted with 2×2 mL of methanol into a glass tube. The eluate was dried in a water bath at 40°C under a stream of nitrogen. The residues were dissolved in 0.2 mL of methanol, and 5 mL of water was added followed by vortexing.

This mixture was cleaned on a ChemElut 1010 column online connected with a Teflon stopcock to a Florisil cartridge. The sample was applied onto the extraction column, and after 10 min, the isoflavones were eluted from the two superimposed columns with 50 mL of a mixture of dichloromethane and ethyl acetate (1:1, v/v) using gravity. The eluate was collected in a rotavapor flask and evaporated under vacuum at 40°C to dryness. The residue was dissolved in 1 mL of methanol and put into a silanized derivatization vial. The methanol was evaporated to dryness at 60°C under nitrogen.

Enzymatic Hydrolysis of Isoflavone Glucuronides.

An enzymatic hydrolysis of the isoflavone glucuronides was used for the total isoflavone content determination in the urine samples. The eluate from the C_{18} column (see Sample Cleanup Procedure) was evaporated to dryness at 40°C under a stream of nitrogen. The residue was dissolved in 0.2 mL of methanol, and 5 mL of a 0.2 mol L^{-1} acetate buffer (pH 4.6) and 50 μL of *H. pomatia* digestive juice was added. The sample was incubated for 90 min at 60°C . After cooling to room temperature, the hydrolyzed urine was cleaned up as described above (Chem Elut plus Florisil). However, the elution was done with 70 mL of a mixture of dichloromethane and ethyl acetate (2:1, v/v).

Derivatization. After the external standard was added, 100 μL of BSTFA containing 1% of TMCS was put into the vial. After vortexing, the solution was heated for 60 min at 60°C . After cooling, 2 μL of this solution was injected into the GC/MS instrument.

Calibration curves were constructed in blank human urine samples.

GC/MS Analysis. The analyses were carried out on a GCQ Finnigan ion trap detector (San Jose, CA), linked to an HP 5890 Series II gas chromatograph Hewlett-Packard (Palo Alto, CA) equipped with an HP-5MS fused silica capillary column (30 m \times 0.25 mm i.d., film thickness = 0.25 μm). Injections were carried out in the splitless mode. The carrier gas was high-purity helium (L'Air Liquide, Liege, Belgium) at a flow rate of 1 mL min^{-1} . The injector and interface temperatures were at 280 and 275 $^\circ\text{C}$, respectively. The oven temperature was programmed from 70 $^\circ\text{C}$ (held for 1 min) to 200 $^\circ\text{C}$ at 40 $^\circ\text{C min}^{-1}$ and then to 280 $^\circ\text{C}$ at 10 $^\circ\text{C min}^{-1}$, the final

temperature being held for 20 min. The temperature of the ion source was 200 $^\circ\text{C}$, and the electron voltage was 70 eV.

RESULTS AND DISCUSSION

A new isolation and cleaning procedure based on SPE (C_{18}) for the isolation of isoflavones from urine samples was developed. The elution of the compounds under study from the C_{18} phase is sufficiently effective with pure methanol. The columns in tandem (ChemElut 1010 and Florisil cartridge) were efficient for a rapid purification of the urine matrix. An advantage of the new purification procedure was an easy regulation of the flow of the elution mixture across the Florisil cartridge by a Teflon stopcock. Other authors (Lu et al., 1995a,b) used ethyl acetate for the elution of isoflavones from the ChemElut column. Our experiments, however, showed that a mixture of dichloromethane and ethyl acetate (1:1, v/v) used on the combined columns gave extracts that were significantly cleaner (results not shown). The exact time between application of sample onto the ChemElut 1010 column and elution thereof was very crucial for the reproducibility of the isolation process. An optimal waiting time of 10 min is recommended. Longer (at most 15 min) waiting times had no further positive influence on the elution. Centrifugation of the urine samples before application on the SPE columns is highly recommended to avoid obstruction.

The GC temperature program was optimized so that separation between the compounds of interest was achieved. The retention times for genistein, daidzein, biochanin A, formononetin (biochanin B), and external standards are summarized in Table 1. The external standards were chosen according to their retention time and the specificity of the mass spectrum of the TMS derivative. A derivatization temperature of 60 $^\circ\text{C}$ and a reaction time of 60 min were used for the TMS ether preparation of all isoflavones and external standards. Derivatization with BSTFA containing 1% of TMCS was fast, simple, and reproducible. As an example, the spectra of daidzein and genistein are shown in Figure 2.

Recovery studies were performed on urine samples spiked at the 5 and 25 ng mL^{-1} level ($n = 5$ for each concentration). External standard was added just before derivatization and the ratios of the area of the sum of the selected ions of the compound of interest to the area of the sum of the selected ions of the external standard were calculated. These values were compared to the ratios obtained for purified urine extracts that were spiked at the 5 or 25 ng mL^{-1} level just before derivatization. The obtained results varied from 63.5 to 89.2% for the 5 ng mL^{-1} level and from 61.3 to 89.6% for the 25 ng mL^{-1} level. Recoveries for the determination of

Table 2. Recovery for Free (5 and 25 ng mL^{-1} Levels) and Total Isoflavones (25 ng mL^{-1} Level) in Urine and Detection Limit for Free Isoflavones^a

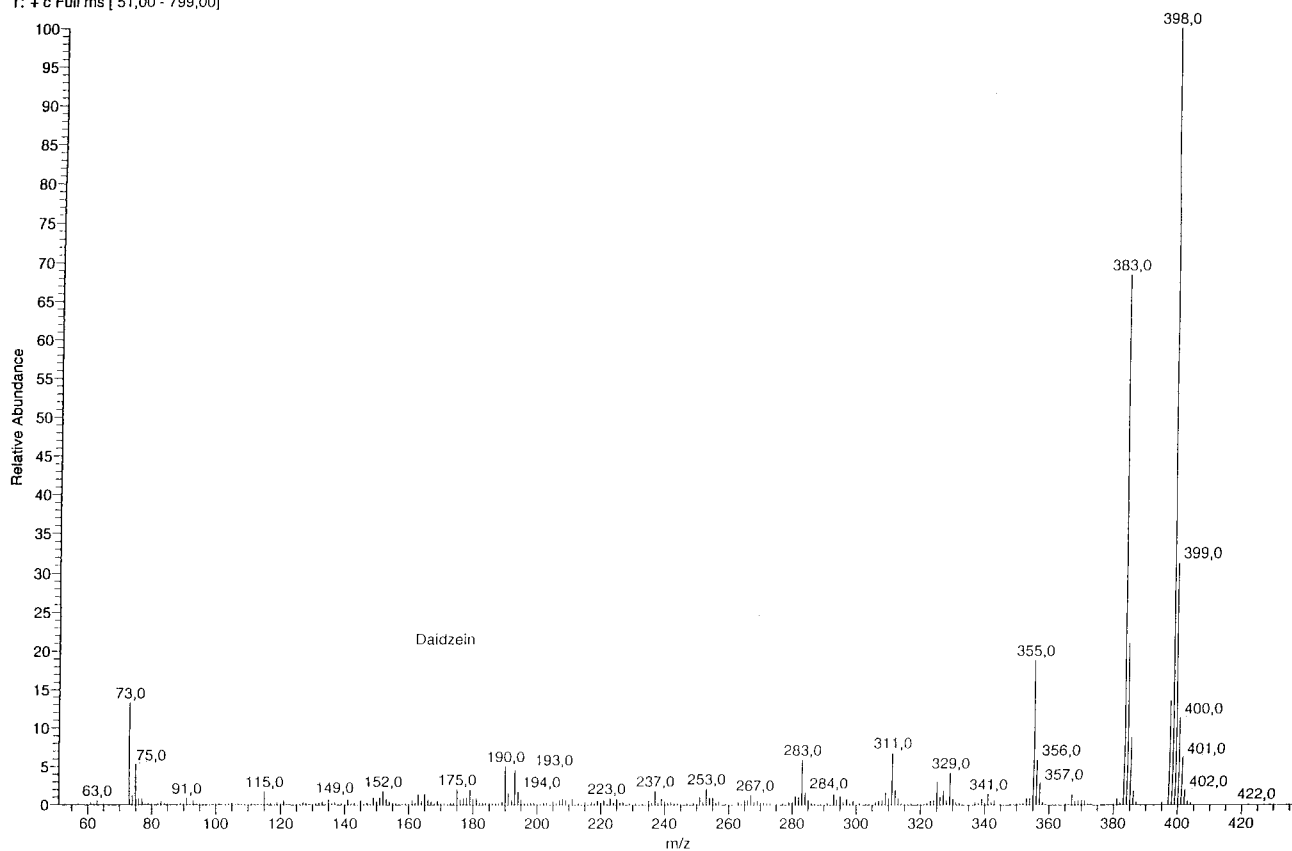
compound	recovery \pm RSD (%), $n = 5$			detection limit (ng mL^{-1}) free isoflavones
	free		total	
	5 ng mL^{-1}	25 ng mL^{-1}	25 ng mL^{-1}	
formononetin	89.2 \pm 30.0	88.5 \pm 9.5	70.6 \pm 11.2	1.05
biochanin A	63.5 \pm 16.6	61.3 \pm 4.2	56.5 \pm 9.7	1.5
daidzein	89.1 \pm 17.2	85.6 \pm 5.6	77.1 \pm 6.2	2.3
genistein	78.6 \pm 21.5	89.6 \pm 13.9	75.9 \pm 7.8	2.2

^a External standard was 6-hydroxyflavone for free isoflavone and robinetin for total isoflavone determination. Recoveries were based on comparison of the ratios of the area of the sum of the selected ions of the isoflavone to the area of the sum of the selected ions of the external standard obtained for a spiked sample to the ratio obtained for a blank matrix spiked just before derivatization.

C:\LCQ\data\ae\gMi1108

11/08/98 13:16:10

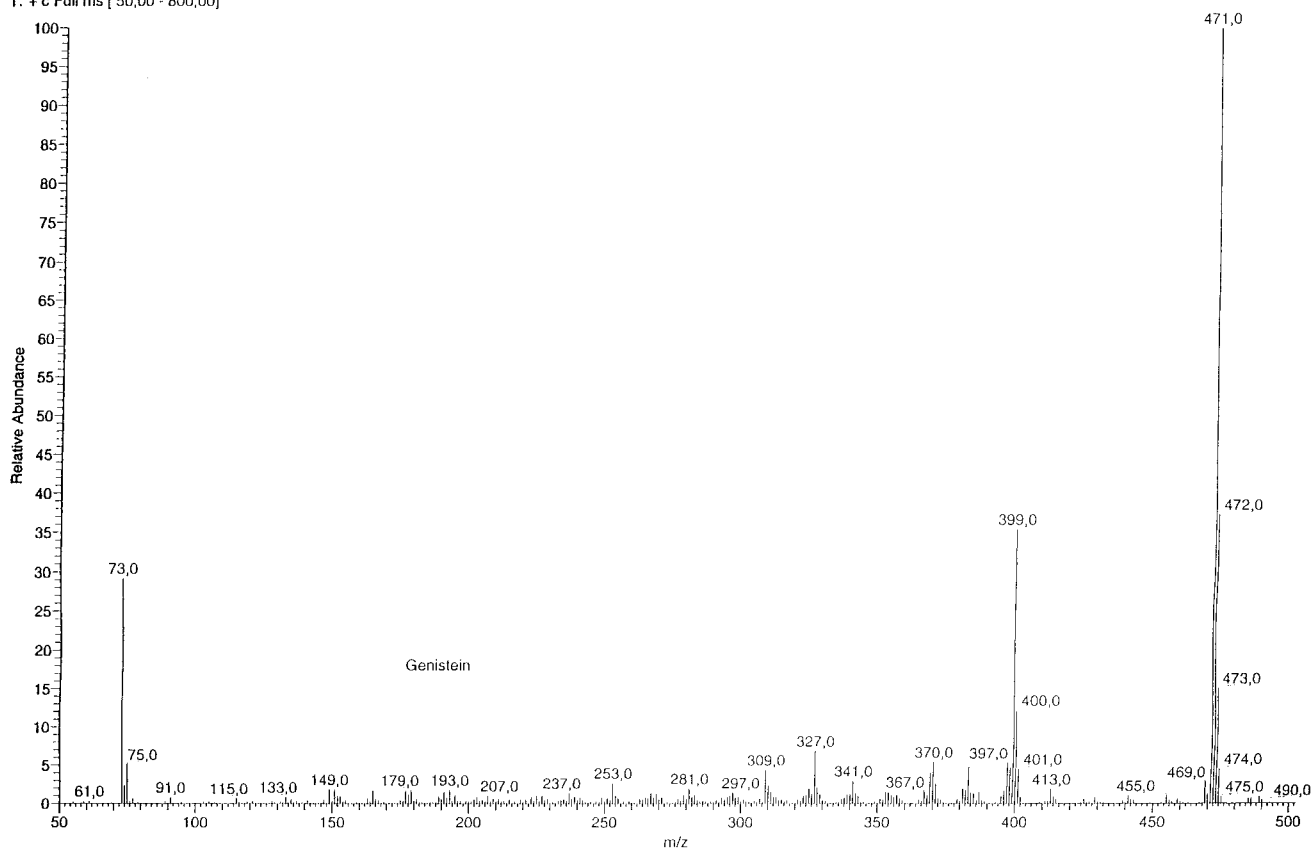
Mixture; Fo, Bio A, Da, Ge, 1 mg/ml, 100

S#: 599 RT: 17.04 AV: 1 NL: 3.05E6
T: + c Full ms [51,00 - 799,00]

C:\LCQ\data\ae\gMi1108

11/08/98 13:16:10

Mixture; Fo, Bio A, Da, Ge, 1 mg/ml, 100

S#: 622 RT: 17.42 AV: 1 NL: 2.18E6
T: + c Full ms [50,00 - 800,00]**Figure 2.** Mass spectra of the TMS derivatives of daidzein and genistein.

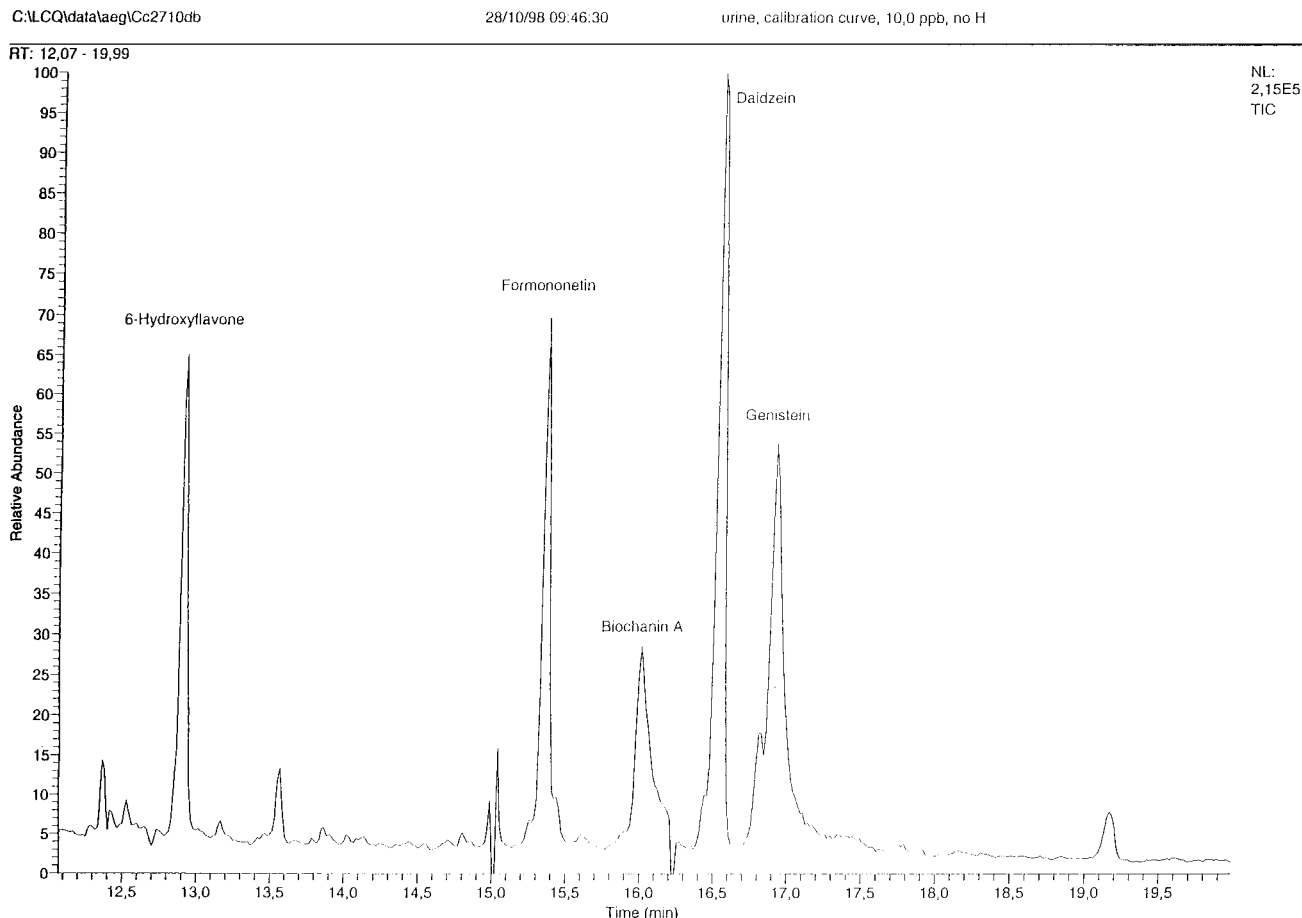


Figure 3. Total ion chromatograms for daidzein, genistein, formononetin, and biochanin A spiked in human urine at a concentration of 10 ng mL⁻¹.

total isoflavones ($n = 5$) after enzymatic hydrolysis (*H. pomatia*) varied from 56.5 to 75.9% for the 25 ng mL⁻¹ level. All results are presented in Table 2. The total ion chromatogram obtained in urine at a concentration of 10 ng mL⁻¹ is shown in Figure 3.

For the determination of the total isoflavone content a hydrolysis was required. Methanolysis (Tang and Crone, 1989) of isoflavone glucuronides with anhydrous hydrogen chloride did not provide extracts that were pure enough for the GC/MS detection, even after cleanup on the combined columns. Therefore, hydrolysis was carried out enzymatically making use of *H. pomatia*.

The external standard 6-hydroxyflavone (ES-1) was suitable for free isoflavone determination, but when using this ES-1 in the procedure in which hydrolysis was necessary, interferences appeared at the retention time of ES-1 in the chromatogram. Robigenin (ES-2), however, had a longer retention time and was selected as ES for the total isoflavone determination.

The limit of detection was determined by analyzing samples fortified with increasing concentrations of the compounds (0, 2.5, 5, 10, and 25 ng mL⁻¹) and a constant amount of external standard. Ratios of the area of the sum of the selected ions of a compound to the area of the sum of the selected ions of the external standard were calculated. A calibration curve was established, and the limit of detection was calculated as $3S_b/m$, where S_b is the standard deviation of the intercept on the y -axis and m is the slope of the calibration curve (Verwaal et al., 1996). Calibration curves were linear, and detection limits ranged from 1 to 2.3 ng mL⁻¹ for

free isoflavones. For the determination of total isoflavones problems with the stability of the standards during the hydrolysis procedure at low concentration were encountered. From the experiments it was concluded that for daidzein and genistein a detection limit of 10 ppb is real. For formononetin and biochanin A this is not possible. Further studies concerning the hydrolysis conditions are necessary to solve this problem.

LITERATURE CITED

- Adlercreutz, H. Phytoestrogens: epidemiology and possible role in cancer protection. *Environ. Health Perspect.* **1995**, 103 (Suppl. 7), 103–112.
- Adlercreutz, H.; Fotsis, F.; Bannwart, C.; Wähälä, K.; Brunow, G.; Hase, T. Isotope dilution gas chromatographic-mass spectrometric method for the determination of lignans and isoflavonoids in human urine, including identification of genistein. *Clin. Chim. Acta* **1991a**, 199, 263–278.
- Adlercreutz, H.; Honjo, H.; Higashi, A.; Fotsis, T.; Hämäläinen, E.; Hagesawa, E.; Okada, H. Urinary excretion of lignan and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet. *Am. J. Clin. Nutr.* **1991b**, 54, 1093–1100.
- Adlercreutz, H.; Fotsis, T.; Lampe, J.; Wähälä, K.; Mäkelä, T.; Brunow, G.; Hase, T. Quantitative determination of lignans and isoflavonoids in plasma of omnivorous and vegetarian women by isotope dilution gas chromatography–mass spectrometry. *Scand. J. Clin. Lab. Invest.* **1993a**, 53 (Suppl. 215), 5–18.
- Adlercreutz, H.; Markkanen, H.; Watanabe, S. Plasma concentrations of phyto-oestrogens in Japanese men. *Lancet* **1993b**, 342, 1209–1210.
- Adlercreutz, H.; Fotsis, T.; Kurzer, M. S.; Wähälä, K.; Mäkelä, T.; Hase, T. Isotope dilution gas chromatographic-mass

- spectrometric method for the determination of unconjugated lignans and isoflavonoids in human feces, with preliminary results in omnivorous and vegetarian women. *Anal. Biochem.* **1995a**, *225*, 101–108.
- Adlercreutz, H.; Goldin, B. R.; Gorbach, S. L.; Höckerstedt, K.; Watanabe, S.; Hämäläinen, E.; Markkanen, H.; Mäkelä, T.; Wähälä, H.; Hase, T.; Fotsis, T. Soybean phytoestrogens intake and cancer risk. *J. Nutr.* **1995b**, *125*, 757S–770S.
- Caragay, A. B. Cancer preventive foods and ingredients. *Food Technol.* **1992**, *46*, 65–68.
- Franke, A. A.; Custer, L. J. Daidzein and genistein concentration in human milk after soy consumption. *Clin. Chem.* **1996**, *42*, 955–964.
- Franke, A. A.; Custer, L. J.; Cerna, C. M.; Narala, K. K. Quantitation of phytoestrogens in legumes by HPLC. *J. Agric. Food Chem.* **1994**, *42*, 1905–1913.
- Franke, A. A.; Custer, L. J.; Wang, W.; Shi, C.-Y. HPLC analysis of isoflavonoids and other phenolic agents from foods and from human fluids. *Proc. Soc. Exp. Biol. Med.* **1998**, *217*, 263–273.
- Hampl, R.; Lapcik, O.; Wähälä, K.; Starke, L.; Adlercreutz, H. Radioimmunoassay analysis of phytoestrogens of isoflavonoid series. *Chem. Listy* **1998**, *92*, 44–50.
- Hutchins, A. M.; Lampe, J. W.; Martini, M. C.; Campbell, D. R.; Slavin, J. L. Vegetables, fruits and legumes: effect on urinary isoflavonoid phytoestrogens and lignan excretion. *J. Am. Diet. Assoc.* **1995**, *95*, 769–774.
- Joannou, G. E.; Kelly, G. E.; Reeder, A. Y.; Waring, M. A.; Nelson, C. A urinary profile study of dietary phytoestrogens. The identification and mode of metabolism of new isoflavonoids. *J. Steroid Biochem. Mol. Biol.* **1995**, *54*, 167–184.
- Kaufman, P. B.; Duke, J. A.; Brielmann, H.; Boik, J.; Hoyt, J. E. A comparative survey of leguminous plants as sources of the isoflavones, genistein and daidzein: implications for human nutrition and health. *J. Alternat. Complement. Med.* **1997**, *3*, 7–12.
- Kelly, G. E.; Nelson, C.; Waring, M. A.; Joannou, G. E.; Reeder, A. Y. Metabolites of dietary (soya) isoflavones in human urine. *Clin. Chim. Acta* **1993**, *223*, 9–22.
- Lapcik, O.; Hampl, R.; Al-Maharik, N.; Salakka, A.; Wähälä, K.; Adlercreutz, H. A novel radioimmunoassay for daidzein. *Steroids* **1997**, *62*, 315–320.
- Lapcik, O.; Hill, M.; Hampl, R.; Wähälä, K.; Adlercreutz, H. Identification of isoflavonoids in beer. *Steroids* **1998**, *63*, 14–20.
- Lu, L.-J. W.; Broemeling, L. D.; Marshall, M. V.; Ramanujam, S. V. M. A simplified method to quantify isoflavones in commercial soybean diets and human urine after legume consumption. *Cancer Epidemiol. Biomarkers Prev.* **1995a**, *4*, 497–503.
- Lu, L.-J. W.; Grady, J. J.; Marshall, M. V.; Ramanujam, S. V. M.; Anderson, K. E. Altered time course of urinary daidzein and genistein excretion during chronic soya diet in healthy male subjects. *Nutr. Cancer* **1995b**, *24*, 311–323.
- Mazur, W.; Fotsis, T.; Wähälä, 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*, 169–180.
- Messina, M.; Barnes, S. The role of soy products in reducing risk of cancer. *J. Natl. Cancer Inst.* **1991**, *83*, 541–546.
- Messina, M.; Persky, V.; Setchell, K. D. R.; Barnes, S. Soy intake and cancer risk: a review of in vitro and in vivo data. *Nutr. Cancer* **1994**, *21*, 113–131.
- Morton, M. S.; Wilcox, C.; Wahlqvist, M. L.; Griffiths, K. Determination of lignans and isoflavonoids in human female plasma following dietary supplementation. *J. Endocrinol.* **1994**, *142*, 251–259.
- Murphy, P. A.; Song, T.; Buseman, G.; Barua, K. Isoflavones in soy-based infant formulas. *J. Agric. Food Chem.* **1997**, *45*, 4635–4638.
- Reinli, K.; Block, G. Phytoestrogen content in foods—a compendium of literature values. *Nutr. Cancer* **1996**, *26*, 123–148.
- Rosenblum, E. R.; Campbell, I. M.; van Thiel, D. H.; Gavalier, J. S. Isolation and identification of phytoestrogens from beer. *Alcohol Clin. Exp. Res.* **1992**, *16*, 843–845.
- Seow, A.; Shi, C.-Y.; Franke, A. A.; Hankin, J. H.; Lee, H.-P.; Yu, M. C. Isoflavonoid levels in spot urine are associated with frequency of dietary soy intake in a population-based sample of middle-aged and older Chinese in Singapore. *Cancer Epidemiol. Biomarkers Prev.* **1998**, *7*, 135–140.
- Tang, P. W.; Crone, D. I. A new method for hydrolyzing sulfate and glucuronyl conjugates of steroids. *Anal. Biochem.* **1989**, *182*, 289–294.
- Verwaal, W.; van Bavel, M.; Boot, A.; Bravenboer, J.; de Goey, F.; Maas, A.; van der Putten, A. Valideren van (fysisch)-chemische en (fysisch)-mechanische methoden op het niveau van de wettelijke eis. *Ware(n)-Chemicus* **1996**, *26*, 17–86.
- Wang, H.; Murphy, P. A. Isoflavone content in commercial soybean foods. *J. Agric. Food Chem.* **1994**, *42*, 1666–1673.
- Wang, W.; Tanaka, Y.; Han, Z.; Cheng, J. Radioimmunoassay for quantitative analysis of formononetin in blood plasma and rumen fluid of wethers fed red clover. *J. Agric. Food Chem.* **1994**, *42*, 1584–1587.

Received for review February 16, 1999. Revised manuscript received June 23, 1999. Accepted July 8, 1999. This work was supported by The National Fund for Scientific Research (Grant G0019.97) and the Belgian Office for Scientific, Technical and Cultural Affairs (DWTC).

JF990159Z