ELISA for Free *S*-equol in Human Urine

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Abstract

Sosvorová L., Lanková P., Bičíková M., Prokudina E.A., Al Malarik N., Lapčík O. (2011): **ELISA for free *S*-equol in human urine**. Czech J. Food Sci., **29**: 57–64.

The popularity of phytoestrogen food supplements of plant origin steadily increases. They are used to solve the problems related to the climacterium. Their efficiency depends on woman's capability to metabolise the active components – isoflavonoids. Women able to convert daidzein to *S*-equol are supposed to get a greater benefit from these supplements. To determine *S*-equol urine levels, the competitive ELISA was developed. Carboxymethylequol coupled to BSA via 4'-*O*-position was used as an immunogen. The assay conditions, including the concentrations of the coating antigen and antisera, were optimised. The detection limit was 0.1 ng/ml (5 pg/well) and 50% intercept was 1 ng/ml (50 pg/well). The intraassay coefficients of variation (*CV*) varied from 4.7% to 9.9%, for the interassay *CV* the corresponding values of 2.6–11.6% were obtained. The recovery of the standard added to urine ranged between 89% and 104%. All examined samples were obtained from women in climacterium. The volunteers had been taking phytoestrogen food supplements for 3 months. The urine samples were collected before and after the therapy. The levels of free urinary *S*-equol after the therapy ranged between 0 ng/ml and 42.4 ng/ml. The equol production was observed in 57% of the examined patients. The laboratory results were confronted with patients' subjective evaluations.

Keywords: isoflavonoids; metabolism; menopause

The isoflavonoids daidzein and genistein are phytoestrogens naturally occurring in legumes. These substances have received increasing attention because of their biological activities, including the reduction of the climacteric symptoms (Cornwell *et al.* 2004), reduction of cardiovascular disease risk (Clarkson *et al.* 2002), positive effect on bone density (Chen *et al.* 2003), and cancer-oprotective effects (Cornwell *et al.* 2004). The richest sources of isoflavonoids are soy (*Glycine max*) and red clover (*Trifolium pratense*). From the biosynthetical point of view, isoflavonoids are produced by plant phenylpropanoid metabolism (Velíšek *et al.* 2008). Isoflavonoid rich soy and red clover extracts are used in food supplements designated for women in climacterium. They take place in the treatment of menopausal symptoms, being considered a good alternative to the conventional hormone replacement therapy (Beck *et al.* 2005). Isoflavonoids naturally occur as glycosides and aglycones. The glycosides are readily hydrolysed in the intestine and the aglycones undergo

Supported by the Ministry of Education, Youth and Sports of the Czech Republic, Project No. MSM 6046137305, and by the Czech Science Foundation, Grant No. 303/08/0958.

further metabolic modifications (Cornwell *et al*. 2004). Part of them are conjugated to glucuronates and sulfates in the liver. Free isoflavonoids undergo intestinal biotransformations, including dehydroxylation, reduction, C-ring cleavage, and demethylation (Setchell *et al*. 2002). All these transformations take place distally, presumably in the colon. The most important transformation is the metabolic conversion of daidzein via dihydrodaidzein to equol or *O*-desmethylangolensin by the action of the intestinal microflora (Joannou *et al*. 1995). Equol is the most active phytoestrogen in contrast to *O*-desmethylangolensin, whose estrogenic activity is rather low (Axelson *et al*. 1982). Marrian and Haslewood (1932) first isolated equol as a contaminant during the hydroxyestrin isolation from a mare's in-foal urine. In human urine, equol was first identified by Axelson *et al*. (1982). The estrogenic activity of equol can be explained by its structural similarity to 17-β-estradiol (Sathyamoorthy & Wang 1997), enabling its binding to the estrogen receptors (Figure 1). The published reports indicate that only about 30–60% of humans are able to produce equol from daidzein (Setchell *et al*. 1984, 2003; Lampe *et al*. 1998; Rowland *et al*. 2000). This observation has led to the terminology of being an "equol producer" or "equol non-producer" (Setchell *et al*. 2002). Intensive research has been performed in order to identify the equol-producing bacteria. Recently two genera of such bacteria have been recognised in human intestinal microbiota, i.e. – *Eggerthella* sp. and *Adlercreutzia equolifaciens* (Maruo *et al*. 2008; Yokoyama & Suzuki 2008), and a new strain *Asaccharobacter celatus* has been discovered in rat intestine (Thaworkuno *et al*. 2009). Equol is a chiral compound with two enantiomeric forms, i.e. *R*- and *S*-equols. Out of them, only *S*-equol is produced by the intestinal microflora (Setchell *et al*. 2005). Muthyala *et al*. (2004) demonstrated that the binding affinity of *S*-equol to estrogen receptor β is 13 times higher than that of *R*-equol and, moreover, that *S*-equol has a much higher estrogenic activity than the other isomer. On the other hand, *R*-equol showed a 4 times higher preference for binding to estrogen receptor α than *S*-equol. It has been proved that almost 50% of equol circulates in the free form. This is substantially higher than the proportions of free daidzein (18.7%) or estradiol (4.6%). As only the unbound fraction is available for the receptor occupancy, this may be responsible for the higher estrogenic potency of equol (Nagel *et al*. 1998).

The analytical methods usually employed for the determination of *S*-equol are HPLC-MS or GC-MS (Setchell *et al*. 2005). Racemic equol can be determined by ELISA employing an equol hapten with spacer arm on the oxygen atom at the C7 position (Bennetau-Pelissero *et al*. 2000). Another metod developed to determine racemic equol is TR-FIA (time-resolved fluoroimmunoassay) method (Brouwers *et al*. 2003). This

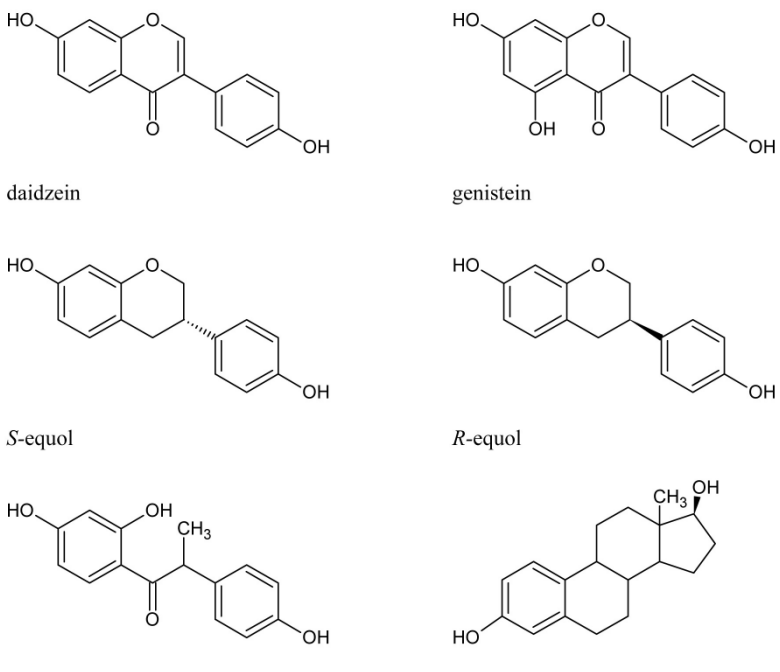

Figure 1. Structures of phytoestrogens and 17-β-estradiol

method displays comparable sensitivity, but the sample preparation is more difficult and the assay formate less disposable.

In the present study, we report a new ELISA method for the estimation of *S*-equol in human urine. ELISA is one of the most convenient analytical methods and is applicable in the majority of laboratories. The sensitivity of our method is higher, the preparation of the samples is easier, and the costs are much lower in comparison with the chromatographic methods.

MATERIAL AND METHODS

Chemicals. Tetramethylbenzidine (TMB), dimethylsulfoxid, hydrogen peroxide, gelatin from porcine skin, citrate-phosphate buffer tablettes, Tween 20, bovine serum albumine (BSA), ovalbumine (OVA), equol, *N,N'*-dicyclohexylcarbodiimide (DCC), and *N*-hydroxysuccinimid (NHS) were purchased from Sigma (St. Louis, USA). The peroxidase labelled swine antibody against rabbit antibody (SwaR/Px) was from Sevapharma a.s. (Prague, Czech Republic). The microtitration plates COSTAR (cat. Nr.: 9018) were supplied by Corning Incorporated (New York, USA). Diethyl ether and methanol were from Merck AG (Darmstadt, Germany). *S*-equol and *R*-equol were from Cayman Chemical Company (Michigan, USA). 4'-*O*-Ethoxymethyldaidzein was prepared as described elsewhere (Al Maharik *et al*. 1999).

Synthesis of 4'-O-carboxymethylequol. 4'-*O*-Ethoxymethyldaidzein was reduced by palladised charcoal and hydrogen gas in ethanol in a hydrogenation apparatus. The hydrolysis of the corresponding ethyl ester (4'-*O*-ethoxycarbonylmethyl-equol) with 10% KOH in aqueous methanol provided the desired 4'-*O*-carboxymethylequol in a good yield (Brouwers *et al*. 2003). Racemic equol was used for the synthesis of equol conjugates, because of nonavailibility of *S*-equol at that time.

Immunogen synthesis and immunisation. The immunogen was synthesised in a reverse micellar system, originally developed for the steroid immunogen synthesis by Yatsimirskaya *et al*. (1993), with minor modifications. Ten milligrams of 4'-*O*-carboxymethylequol was left to react overnight with DCC and NHS (molar ratio 3:4:5) in anhydrous dimethylformamide at room temperature. The reaction mixture was centrifuged in order to remove the crystals of dicyclohexylurea. The supernatant was used for conjugation with BSA for immunogen and with OVA for immobilised antigen synthesis. The starting molar ratio equol:protein was 50:1. Protein was dissolved in 0.01 mol/l sodium-bicarbonate buffer pH 8.5. This solution was added dropwise to dioctylsulfosuccinate in octane under continuous stirring to form the reverse micellar phase. After the mixture became transparent, the dimethylformamide solution of the active intermediate, formed from 4'-*O*-carboxymethylequol, was added. This mixture was stirred for 24 h at room temperature. The equol-protein conjugate was isolated from the mixture by precipitation with cold acetone and centrifuged once again. The sediment was dissolved in distilled water, filtered through a 0.22 µm Millipore filter, and lyophilised. The rabbits were immunised and the sera were collected using a standard procedure (Brouwers *et al*. 2003).

ELISA of S-equol. The assay was performed using an indirect competitive format with immobilised antigen. First, the equol-OVA conjugate was immobilised on the wells of the microtitration plate. The stock solution was diluted with carbonate-bicarbonate buffer (0.05 mol/l, pH 9.6) and dosed to the wells of the microtitration plate in the amount of 100 µl/well. The plate was incubated overnight at 4°C. All wells were rinsed four times with 300 µl/well PBS-Tween (0.01 mol/l phosphate buffer, pH 7.4; 0.05% Tween 20) after the incubation. After that, the wells were saturated with 100 µl of 1% water solution of gelatine, in order to saturate the remaining binding places. The plate was incubated for 30 min on an ELISA shaker and rinsed four times with 300 µl/well of PBS-Tween. The calibration curves were constructed by means of serial dilutions of the standards in water starting from 312.5 ng/ml. The samples and standards were added in the amount of 50 µl/well and then the primary antibody was added in the same volume. Each plate was incubated for 1.5 h at room temperature on an ELISA shaker. The plates were rinsed four times with 300 µl/well PBS-Tween. The quantification of the captured primary antibody was performed with a peroxidase labelled secondary antibody (100 µl SwaR/Px/well diluted 1:4000 in PBS-Tween). After one hour incubation, the plates were rinsed four times with 300 µl/well PBS-Tween. To measure the peroxidase activity, 100 µl/well of the substrate solution containing 0.02% H_2O_2 and 0.01% TMB in citrate-phosphate buffer (0.1 mol/l, pH 5.0) were added. The reaction

took place at room temperature for 10 min and was stopped with 50 µl/well of H_2SO_4 × 2 mol/l. The absorbance was read at 450 nm on DSX System from Thermo Labsystems.

The calibration curve was created as the dependence of absorbance on the logarithm of antigen concentration (pg/well) (Vítková *et al*. 2004).

The cross-reactions were calculated as a percentage using the equation:

$$CR(AG2) = \frac{I_{50}(AG1)}{I_{50}(AG2)} \times 100 \quad (\%)$$

where:

$I_{50}(AG1)$ – 50% intercept of the calibration curve obtained for the corresponding immunogen

$I_{50}(AG2)$ – 50% intercept of the calibration curve of the cross-reacting antigen

Effect of sample matrix. Standard curves were prepared in 1%, 5%, and 20% urine and compared with the calibration curves diluted in water in order to test the effect of the sample matrix. Therefore, the curves diluted with 5% and 20% urine were shifted. To overcome this problem, the extraction of the analyte into diethyl ether was chosen.

Sample extraction. 250 µl of urine was diluted with 250 µl of water and extracted with 1 ml of diethyl ether. The water phase was frozen in solid carbon dioxide and the ether phase was transferred into a glass tube. The solvent was evaporated and the dry residue was dissolved in 1 ml of 0.01M PBS (0.01 mol/l phosphate buffer, pH 7.4), thus 200 µl of the solution corresponded to 50 µl of the sample. The standard curve prepared in the same assay buffer was processed in the same way as the samples. For the HPLC-MS, 1 ml of urine was extracted with 2 ml of diethyl ether. The water phase was frozen in solid carbon dioxide and the ether phase was transfered into a glass tube. The solvent was evaporated and the dry residue was dissolved in 50 µl of methanol-water (40:60), 50 µl of the solution thus corresponded to 1 ml of the sample.

Determination of S-equol by HPLC-MS. The HPLC-MS system consisted of an Hewlett Packard HP/Agilent Technologies (USA) 1100 HPLC series and an HP Mass selective detector (G1946A) and was controlled by ChemStation software (revision A 07.01). A LiChroCART® 125-4 mm Purospher® STAR RP-18 endcaped (5 µm) HPLC-Cartridge was used as the column (Merck, Darmstadt, Germany). The mobile phases were 40% methanol (solvent A) and 100% methanol (solvent B) both containing 0.5% acetic acid. The following gradient was employed (all steps linear): 0 min 100:0 (A:B); 5 min 80:20; 15 min 55:45; 20 min 0:100; 23 min 0:100; 27 min 100:00, and at 27 min stop followed by 3 min post-run. The flow-rate was 0.8 ml/min, the temperature of the column thermostate was set at 25°C and the injection volume was 10 µl. The mass spectrometer was operated in the positive ESI mode and the nebuliser gas pressure was 60 psi. The drying gas was used at a flow-rate of 12 l/min and temperature of 300°C, and the capillary voltage was 3.5 kV. *S*-equol was identified by comparing its retention time (t_R) and molecular ion $[M+H]^+$ with those of the standard. The detection limit for equol was with this method 0.01 µg for 10 µl injection. The detection limit was calculated based on the three times the signal-to-noise ratios.

Subjects. Morning urine samples were obtained from 28 patients of the Institute of Endocrinology in Prague. The samples were frozen and stored at –18°C. All subjects were in menopause and their serum levels of LH, FSH, and estradiol were in accordance with the menopausal status. All volunteers had common dietary habits and agreed to take commercial phytoestrogen food supplements designated for women in climacterium. The supplements were based on the extracts from red clover or soy and each pill contained 40 mg of isoflavonoids. The volunteers took one pill twice a day for three months. Morning urine samples were collected before and after 3 months of therapy. Written informed consent was obtained from all subjects.

RESULTS AND DISCUSSION

Immunoassay optimisation

First, in this experimental study, the reagents concentrations were preliminary optimised to satisfy the following assay criteria: good sensitivity, minimum immunoreagent consumption, and sufficient chromatic signal. Several amounts of the equol-OVA conjugate immobilised on the wells of the microtitration plate (0.01–0.25 µg/ml) against different amounts of primary antibody (3–80 µg/ml) were investigated in the assays of standard solutions containing *S*-equol at concentrations of 0, 0.1, 0.5, 2.5, 12.5, 62.5, and 312.5 ng/ml, respectively. Subsequently, some other factors affecting the assay performance were also evaluated as follows:

the type of microtitration plate, the type of assay buffer, the incubation time, and the incubation temperature. Moreover, additives such as BSA (0.1%), gelatine (0.1%), and their combinations with and without the surfactant Tween 20 (0.05%), were tested to improve further the assay performance. Optimised conditions were chosen by means of the spectrofotometrical evaluation of the microtitration plates as a compromise between sufficient chromatic signal, minimum reagent consumption, and good assay sensitivity (50% intercept – I_{50}, detection limit). All these conditions were optimal at the antibody concentration of 8 µg/ml and conjugate concentration of 0.1 µg/ml.

Reliability of the assay and parameters of calibration

The detection limit for *S*-equol was 0.1 ng/ml (5 pg/well) and 50% intercept was 1 ng/ml (50 pg per well). Five urine samples were analysed in 5 days for the determination of intra- and interassay coefficients of variation. The intraassay coefficients of variation, determined from sixplicate parallel analyse, varied from 4.7% to 9.9% (Table 1). For the interassay coefficients of variation (Table 1), the corresponding values 2.6–11.6% were obtained.

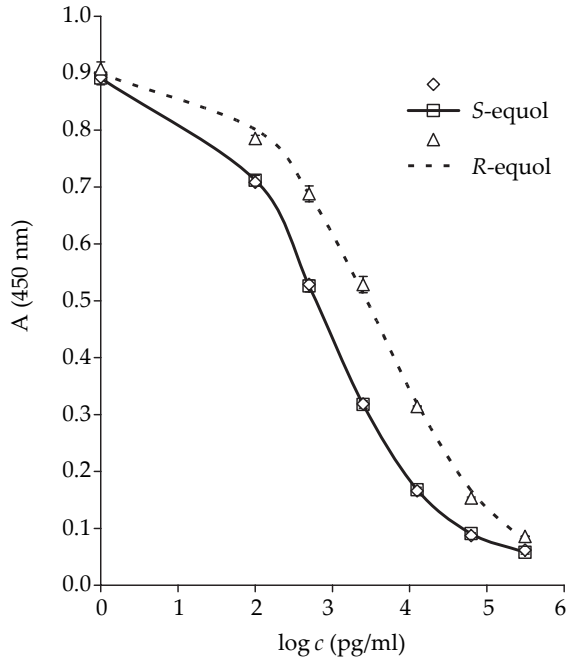

Figure 2. Standard curves for *S*-equol and *R*-equol. Sensitivity of the ELISA to *S*-equol is more than five times higher than to *R*-equol

Table 1. Intraassay and interassay coefficients of variation of *S*-equol

	Intraassay		Interassay	
Sample	(ng/ml)	*CV* (%)	(ng/ml)	*CV* (%)
5B	55.1	4.7	45.9	11.6
4B	32.4	9.9	28.0	2.6
11	19.2	4.6	16.8	6.5
32B	3.1	5.0	3.4	10.8
29B	0.8	6.9	0.9	5.3

The recovery of the standard added to urine ranged from 89% to 104%.

Specificity of the antiserum

Cross-reactivities of the selected isoflavonoids, flavonoids and steroid hormones are summarised in Table 2. As expected, the antiserum reacted with both equol enantiomers. However, its sensitivity to *S*-equol was more than five times higher (Figure 2).

Free *S*-equol levels in urine

The average basal concentration of free *S*-equol in the urine of 28 patients ranged from zero to 0.4 ng/ml (in average 0.12 ng/ml). The distribution of basal levels is shown in Figure 3. After 3 months of phytoestrogen therapy, *S*-equol levels ranged from zero to 42.4 ng/ml (in average 6.25). Sixteen out of the 28 women (57%) had free *S*-equol urine levels higher than 1 ng/ml. These subjects were considered to produce *S*-equol. This level was es-

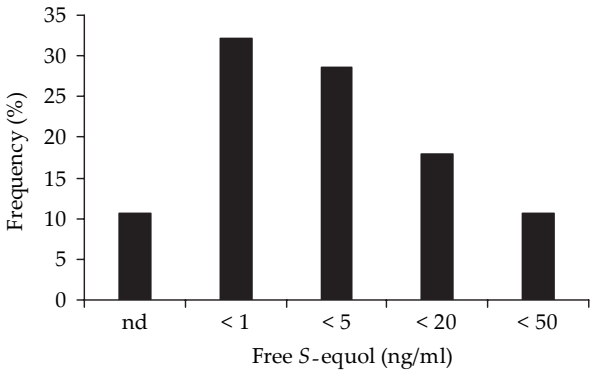

Figure 3. Frequency distribution of free ether extractable *S*-equol urine levels (n.d. = not detectable)

Table 2. Cross-reactivities

Compound	Cross-reactivity (%)
S-equol	100.00
R-equol	17.50
7,3',4'-trihydroxyisoflavone	0.11
6,7,4'-trihydroxyisoflavone	0.05
7,4'-dimethoxyisoflavone	0.10
5,7,4'-trimethoxyisoflavone	0.03
7-hydroxyisoflavone	0.06
5-OH,7,4'-dimethoxyisoflavone	< 0.01
Coumestrol	< 0.01
Glycitein	0.14
Daidzein	0.10
Dihydrodaidzein	0.04
O-demethylangolensin	< 0.01
Genistein	0.03
Formononetin	< 0.01
Biochanin A	0.04
Kaempferol	0.05
Naringin	0.05
Naringenin	0.03
Naringenin-7-glc	0.10
Apigenin	0.02
Cortisol	< 0.01
Testosterone	0.03
Epitestosterone	< 0.01

tablished according to the decrease of climacteric symptoms by the Kupperman index using a standardised questionary. (Wiklund *et al*. 1992).

Correlation between ELISA and HPLC-MS

The correlation between ELISA and HPLC-MS was determined. Twenty eight urine samples with *S*-equol contents from zero to 42.4 ng/ml were analysed using ELISA and HPLC-MS. For ELISA, duplicate determinations of the samples were used. For HPLC-MS, the values were based on single determinations. The sensitivity of HPLC-MS was lower than that of ELISA. However, the results correlated satisfactorily for the urinary *S*-equol levels above 10 ng/ml (Figure 4).

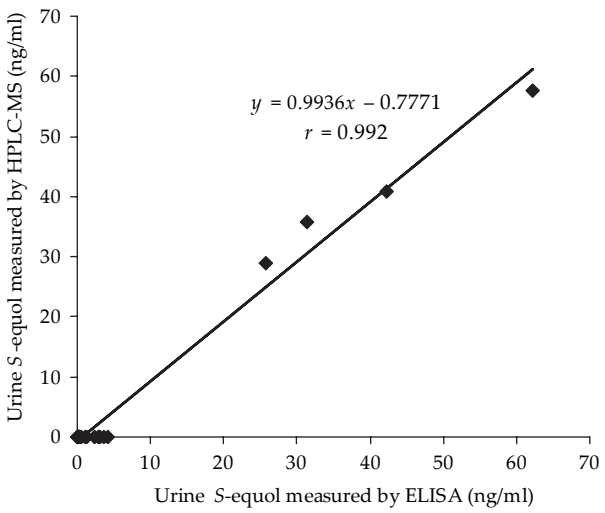

Figure 4. Correlation between results obtained from analyzing 28 urine samples using the ELISA and HPLC-MS

Comparison with other methods

Previously, an ELISA technique for the estimation of equol was developed, employing an equol hapten with spacer arm on the oxygen atom at the C7 position (Bennetau-Pelissero *et al*. 2000). This method was calibrated with racemic equol and was by an order of magnitude less sensitive than the ELISA based on the equol-4'-*O* conjugates reported in this study. Previously, we developed also an ELISA employing the haptens with spacer arm at the C7 position, but this method was less sensitive as well (Bukáčková 2008). The 4'-position based haptens were used also in the TR-FIA (time-resolved fluoroimmunoassay) method for equol published by Brouwers *et al*. (2003), which displayed a comparable sensitivity. *S*-equol levels can also be measured by means of HPLC-MS or GC-MS, but with lower sensitivity than is that of our ELISA and at a higher cost (Setchell *et al*. 2005).

In this study, we did not hydrolyse the urine samples using β-glucuronidase aryl sulfatase from *Helix pomatia* (Bennetau-Pelissero *et al*. 2003; Mathey *et al*. 2006) in order to simplify and speed up the ELISA as much as possible. Since *S*-equol circulates in 49.7% in the unbound form in human fluids (Nagel *et al*. 1998), hydrolysis was not necessary. Moreover, the free *S*-equol urine level reflects the real effective phytoestrogen potential. Due to this step, we accelerated the analysis by two days. For the preparation of urine samples, we used only a simple extraction into diethyl ether. We also tried to measure

S-equol levels in diluted native urine, but the sample matrix effect was very strong. By ELISA, we managed to determine the free *S*-equol urine levels within a few hours. Therefore, equol producers and non-producers can be easily distinguished. The urine level 1 ng of *S*-equol/ml was determined as the threshold. The volunteers with urine *S*-equol concentration higher than is the above mentioned level were marked as the equol producers. All the equol producers benefited from the phytoestrogen therapy by a decrease of their subjective climacteric symptoms as confirmed by a standardised questionnaire.

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Received for publication April 13, 2010
Accepted after corrections September 7, 2010

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