Acta Poloniae Pharmaceutica – Drug Research, Vol. 71 No. 2 pp. 265–269, 2014 ISSN 0001-6837
Polish Pharmaceutical Society

ANALYSIS OF COMPOUNDS WITH PHYTOESTROGENIC ACTIVITY IN DIETARY SUPPLEMENTS WITH USE OF HPTLC-DENSITOMETRY METHOD

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Abstract: Soy (*Glycine max* L., Fabaceae) and soy products are becoming more popular because of their low toxicity and therapeutic effects. Soy possesses antioxidant, anti-inflammatory and anti-allergic properties, however, the most important is its estrogenic activity associated with occurrence of phytoestrogens. Isoflavones with phytoestrogenic effects were determined in four commercially available soya formulations. Analyses were performed with the use of high performance thin-layer chromatography (HPTLC) combined with densitometry. The compounds were extracted, hydrolyzed in order to obtain aglycone forms and separated on HPTLC silica gel 60 F_{254} plates with the use of mobile phase consisting of chloroform – ethyl acetate – formic acid 4 : 6 : 0.1 (v/v/v). After drying, the spots on the plates were determined in absorbance/reflectance mode at a wavelength of 260 nm using a computer-controlled densitometer Desaga CD 60.

Keywords: phytoestrogenes, isoflavones, daidzein, genistein, densitometry

Recently, dietary supplements, particularly derived from plants have gained an increasing popularity as an alternative treatment for their low toxicity and therapeutic effects. Soy (*Glycine max* L., Fabaceae) and soy products are a good example of these types of natural preparations. They are commonly used in the treatment of cardiovascular diseases, menopausal complaints, some types of cancer and for the prevention of osteoporosis (1, 2). Traditional diet based on soy may be responsible for the low breast cancer cases in the region of Asia (3, 4). Soy is a rich source of many valuable compounds including proteins, lipids, saponins, trypsin inhibitors, fiber, phytic acid; however, components with estrogenic activity such as isoflavones and cumestrol are the most important from the point of view of health benefits as alternative to the synthetic estrogen receptor modulators commonly used in hormone therapy (4). There are a lot of various preparations of soy available on the Polish market. The most of them are classified as dietary supplements and not as drugs. Therefore, manufacturers are not obliged to confirm quantities of the active ingredients in the product. Concentration of compounds in plants strongly depends on genotypes, growing seasons and environmental effects. For example, total isoflavones amount in soy samples can ranged from 140 to 748 μg/g and from 258 to 1137 μg/g before and after hydrolysis, respectively (5).These results suggest that quality control of natural products is a necessary process.

The most widely employed method for analysis of isoflavones is HPLC (6–8) but it is time consuming and requires large amounts of solvents during the whole chromatographic run.

On the other hand, HPTLC has been an inexpensive and environmental friendly tool for the phytochemical assessment of plant extracts and herbal drug formulations.

The aim of the present work was to elaborate chromatographic conditions for the quantification of the major constituents with phytoestrogenic effects such as isoflavones in soya formulations with use of HPTLC method and comparison of their contents in the most popular commercially available products.

EXPERIMENTAL

Materials and chemicals

Four different preparations of *Glycine max*: Menoplant Soy-a 40+ (ASA Sp. z o.o), Soya meno (Medana Pharma, Terpol Group S.A.), Menostop

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(Hasco-Lek) and Soyfem (Biofarm) were purchased from a local pharmacy. Isoflavones standards: genistein and daidzein were supplied by Sigma-Aldrich (Germany).

All solvents and reagents were at least pro analysis grade from Polish Reagents (POCh, Gliwice, Poland). HPTLC plates were from Merck (Darmstadt, Germany).

Standards and samples preparation

Ten tablets or capsules of each preparation were accurately weighted. An equivalent of one tablet or content of one capsule (Sample 1: Menoplant Soy-a 40+ = 0.3918 g; Sample 2: Soya meno = 0.4613 g; Sample 3: Menostop = 0.2769 g; and Sample 4: Soyfem = 0.3351 g) was twice extracted with methanol (2 × 50 mL) within 30 min. at room temperature in an ultrasonic bath. The obtained extracts were combined and concentrated to 25 mL.

Hydrolysis condition

A volume of 0.8 mL of 1.0 mol/L hydrochloric acid was added to 10 mL of each extract and made up with methanol to 25 mL in volumetric flask. Hydrolysis was conducted during 2 h at 37°C. Before application, all samples were neutralized.

A stock solutions at concentration of 100 mg/mL and 25 mg/mL for daidzein and genistein, respectively, were prepared in methanol.

Chromatography

Chromatography was performed on 20 cm × 10 cm HPTLC silica gel 60 F_{254} plates. The plates were washed with methanol and dried in a stream of hot air before use. Volumes of 2, 4, 6, 8, 10, 12 mL of standard solution of daidzein; 3, 6, 9, 12, 15, 18 mL of standard solution of genistein; 6 mL of samples no 2, 4; 10 of sample no. 1 and 16 mL of sample no. 3 were spotted as 5 mm bands using an automatic applicator Desaga AS 30 (Heidelberg, Germany) under nitrogen at 2.5 atm (track distance: 9 mm, distance from the left edge: 13 mm).

The plates were developed with the mixture of chloroform – ethyl acetate – formic acid 4 : 6 : 0.1 (v/v/v) to a distance of 80 mm in chromatographic chamber DS (Chromdes, Lublin), previously saturated with vapors of the mobile phase. After drying in the stream of warm air, the plates were analyzed by densitometric scanning (Desaga CD-60,

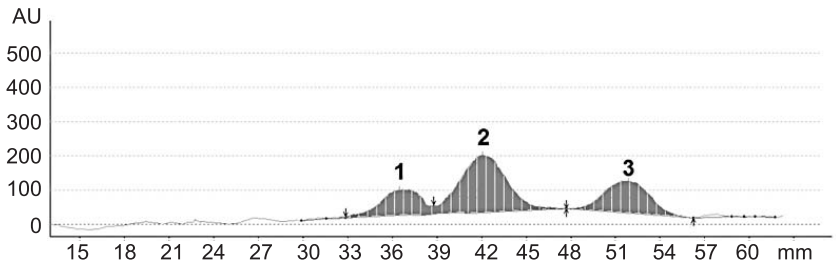

Figure 1. The example of densitogram of hydrolyzed extract from Sample 1: Menoplant Soy-a 40+ obtained at λ = 260 nm; 1 – glycitein, 2 – daidzein, 3 – genistein

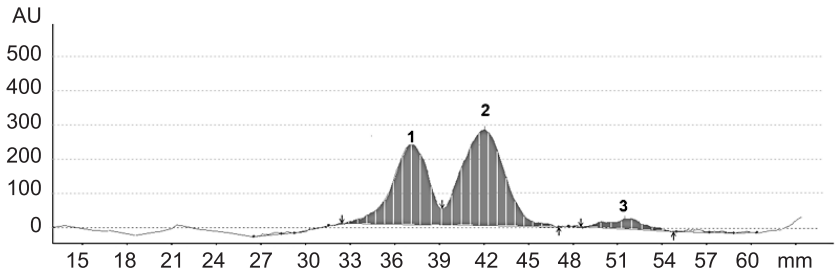

Figure 2. The example of densitogram of hydrolyzed extract from Sample 2: Soya meno obtained at λ = 260 nm; 1 – glycitein, 2 – daidzein, 3 – genistein

Table 1. Summary of validation data for determination of isoflavones.

Validation parameter	Genistein	Daidzein
Linearity range	75–450 ng/spot	200–1200 ng/spot
Regression equation	1861x – 14.6	1240x + 115
Correlation coefficient (R^2)	0.9969	0.9990
Limit of detection (LOD)	9.8 ng/spot	14.2 ng/spot
Limit of quantification (LOQ)	29.8 ng/spot	43.1 ng/spot
Precision (% RSD)	2.5–4.5%	2.7–4.7%
Recovery (%)	98.7%	99.2%

Table 2. The comparison of isoflavones contents in pharmaceutical products.

Pharmaceutical preparation	Genistein [mg/g] ± SD	Daidzein [mg/g] ± SD	Glycitein [mg/g] ± SD	Total amount average [mg/g]
Menoplant Soy-a 40+	5.56 ± 0.17	9.84 ± 0.34	3.45 ± 0.10	18.84
Soya meno	1.70 ± 0.07	13.85 ± 0.40	10.30 ± 0.33	25.85
Menostop	1.10 ± 0.05	4.74 ± 0.22	4.24 ± 0.11	10.07
Soyfem	17.00 ± 0.43	20.32 ± 0.55	14.08 ± 0.35	51.40

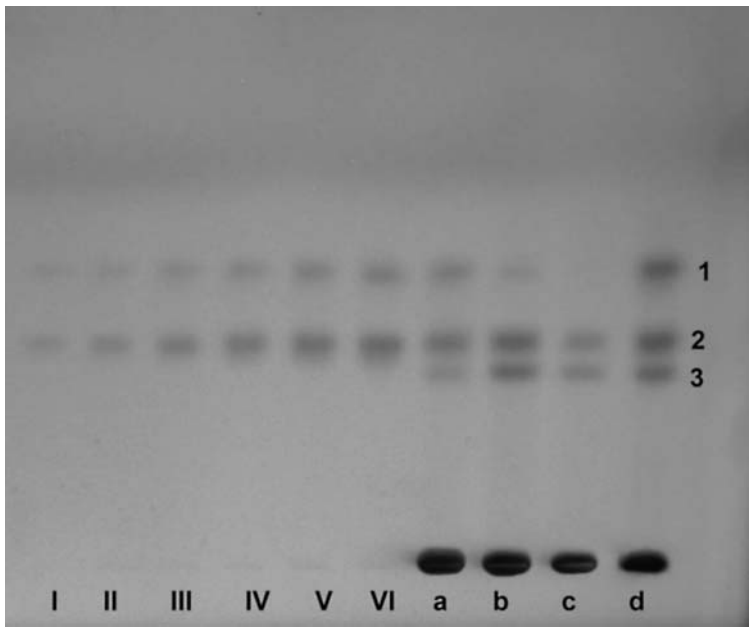

Figure 3. The photograph of HPTLC plate: I – VI calibration curve; a – Menoplant Soy-a 40+, b – Soya meno, c – Menostop, d – Soyfem; 1 – genistein, 2 – daidzein, 3 – glycitein

Heidelberg, Germany) in absorbance/reflectance mode at $\lambda = 260$ nm.

RESULTS AND DISCUSSION

Positive health effects documented for *Glycine max* preparations including alleviating menopausal symptoms in women (4, 9) are attributed to the presence of natural compounds such as glycitein, genistein and daidzein with estrogenic properties (10). Additionally, these isoflavones stimulate osteoblastic bone resorption and protect against osteoporosis (5).

β-Glucosides and acetate or malonyl esters are the predominant forms of isoflavones present in soy

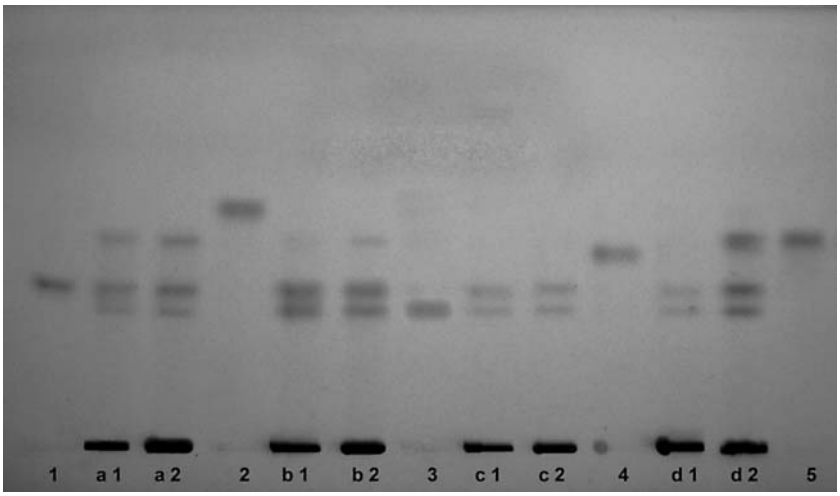

Figure 4. The photograph of HPTLC plate: 1 – daidzein, 2 – biochanin A, 3 – glycitein, 4 –coumestrol, 5 – genistein; a – Menoplant Soy-a 40+ (a1 – before, a2 – after hydrolysis), b – Soya meno (b1 – before, b2 – after hydrolysis), c – Menostop (c1 – before, c2 – after hydrolysis), d – Soyfem (d1 – before, d2 – after hydrolysis)

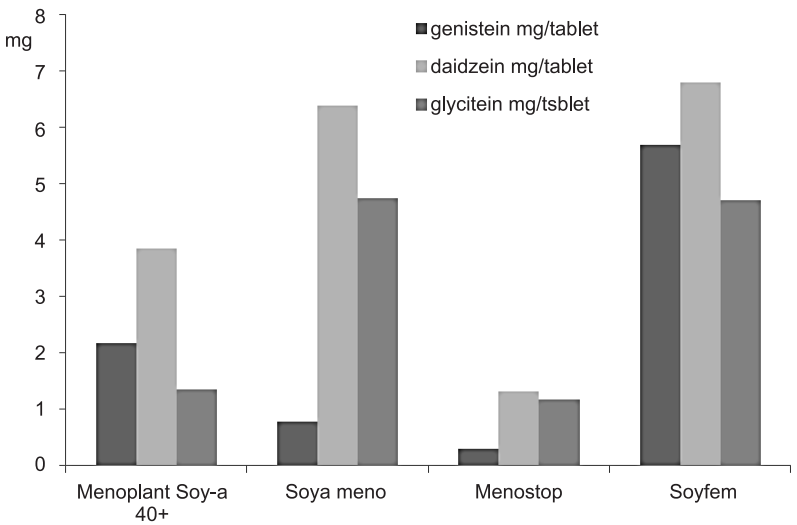

Figure 5. Comparison of isoflavones contents in soy formulations

products, however, free aglycones formed during enzymatic hydrolysis are the bioactive forms (11–13); thus, our studies focus on determination of isoflavones after acid hydrolysis. In our investigations, the hydrolysis parameters such as acidity (pH = 1.5), temperature (T = 37°C) and time (t = 2 h) were similar to physiological conditions of gastric fluid.

Usually, total amount of isoflavones is given by the manufacturers; however, the concentration of genistein, daidzein and glycitein is especially important because of their estrogenic activity. Thus, in our investigation particular attention on these compounds was paid.

In preliminary chromatographic investigations, the mobile phase composition was optimized. The best results were obtained for the mixture of chloroform – ethyl acetate – formic acid 4 : 6 : 0.1 (v/v/v). The bands of analyzed compounds were dense, compact and well separated from the accompanying components (Figs. 1, 2).

The identification was done on the basis of the R_f values. The purity of the peaks in the sample was

ascertained by comparison of absorption spectra with those obtained from the standards. The example of chromatographic plates with standards at different concentrations and analyzed samples is presented in Figure 3.

The method was validated for linearity, precision and accuracy. A calibration plot was established by analysis of standard solution at six different concentrations in the ranges: 0.20–1.2 µg/spot for daidzein and 0.08–0.45 µg/spot for genistein. The amount of glycitein was calculated on the basis of calibration parameters for daidzein as a reference compound because of the high prices and low availability of glycitein standard. The mean peak areas (n = 5) were taken for the construction of calibration curve. The data were analyzed by linear regression least square model and showed a good linear relationship over the tested range.

The accuracy of the method was established by performing recovery experiments at two different levels. Known amounts of genistein (3 and 6 mg) and daidzein (3.5 and 7 mg) were added to the extract before hydrolysis and analyzed as described in Experimental section. The recovery was calculated on the basis of differences between the amount added and quantified. The average recovery was 98.7% for genistein and 99.2% for daidzein. The validation data are summarized in Table 1.

Various concentrations of daidzein, genistein and glycitein were observed in all tested preparations (Table 2). The highest total amount of selected isoflavones (51.40 mg/g) was determined in Soyfem, which is registered as a drug. In this case, the obtained results are close to the total amount of isoflavones given by manufacturer. For the dietary supplements, determined amount of daidzein, genistein and glycitein are much lower than declared isoflavones content. This fact can be explained by occurrence in these preparations the other group of isoflavones or their derivatives, which are not hydrolyzed under condition described in Experimental section. The lowest amount, both of glycitein, genistein and daidzein was quantified in Menostop. It was also noticed that only in the case of Soyfem concentration of aglycones increased significantly after acidic hydrolysis. The differences between extracts before and after hydrolysis are presented in Figure 4.

The comparison of contents of the investigated compounds in one tablet/capsules is given in Figure 5.

The described HPTLC-densitometry method is simple, low cost and fast technique and can be used for routine control of herbal preparation containing isoflavones with phytoestrogenic activity.

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Received: 26. 06. 2013