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Composition in Isoflavones of a Soybean Enzymatic Extract

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Abstract. Soybeans are a main source of plant lipoxygenases and also important sources of isoflavone, including free genistein, daidzein, glycitein and their acetyl, malonyl and glucosilated forms. The enzymatic extract from soybeans (LE) rich in lipoxygenase was checked for the presence of polyphenols through LC-UV-DAD and LC-ESI –MS analysis. LE contains malonyl daidzin and genistein but the amounts in which these are present are very low.

Keywords: isoflavones, daidzein, genistein, enzymatic extract, LC-MS

INTRODUCTION

Isoflavones are a class of phytoestrogens—plant-derived compounds with estrogenic activity. Isoflavones are produced almost exclusively by the members of the Fabaceae/Leguminosae (bean) family. Isoflavones are phenolic compounds containing flavone nucleus, which is composed of 2 benzene rings (A and B) linked through a heterocyclic pyrane C ring. The position of the B ring discriminate flavonoid flavones (C2-position) from isoflavones (C3-position) (Vicaş et al, 2009). Soybeans are important sources of isoflavone levels (Song et al, 1998), present as 12 derivatives, including free genistein, daidzein, glycitein and their acetyl, malonyl or glucosilated forms and also a main source of plant lipoxygenases.

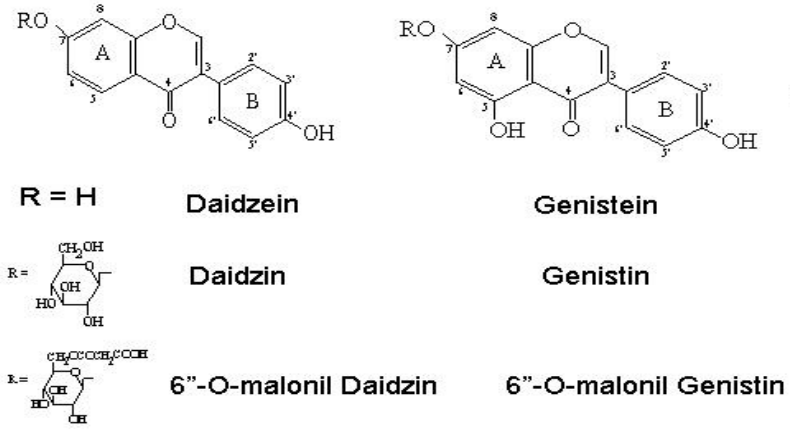

Fig.1. The chemical formula of some isoflavones: genistein, daidzein and their glucosilated and malonyl forms.

The impact of dietary isoflavones, respectively daidzein and genistein, on the health of adults and infants is well documented. An increasing interest for these compounds is registered due to their biological effects including: estrogen-like activity, antioxidant activity (Arora et al, 1998; Patel et al, 2001; Malencić et al, 2007; Sakthivelu et al, 2008) prevention of breast (Warri et al, 2008), prostate (Wang, 2002; Matsumura et al, 2008) and colon cancer (Mac Donald et al, 2005), prevention of heart disease (Lichtenstein 1998; Xiao 2008) of menopausal symptoms and osteoporosis (Potter et al, 1998; Messina, 2007; Ma et al, 2008).

In this study the enzymatic extract from soybeans (LE) was analyzed through LC-UV-DAD and LC-ESI -MS and the quantitative analysis of its components was also performed.

MATERIALS AND METHODS

Extraction of lipoxygenases from soybeans (LE)

To obtain the lipoxygenase extract (LE) an aliquot of 5 g soybean meal was mixed with 30 mL of PBS, pH 7.0, and stirred for 1 h at room temperature. It was then filtered through cheesecloth and centrifuged for 10 min at 16.000 rpm. The supernatant represents the raw extract LE.

Extraction of phenolic compounds by Solide Phase Extraction (SPE)

In brief, 1.500 ± 0.001 g of polyphenolic extract were weighed into pear shaped flasks, and 0.500 ml of the internal standard solution (0.01 mg/ml of methanolic solution of *ortho*-coumaric acid) was added. The solvent was evaporated (in rotary evaporator at 30 ºC under vacuum) and the samples were dissolved in 10 ml of methanol. The extraction was performed using diol-bonded SPE cartridges (GracePure SPE Diol 500mg/3ml) previously conditioned with 6 ml methanol. The phenolic fraction was eluted with 10 ml methanol. After evaporating the solvent in a rotary evaporator at t=30°C, the residues were reconstituted in a 500µl methanol:water 1:1, v/v) and filtered over a 0.45 µm pore size filter for LC-MS analysis (PE). Vials were covered with aluminum foil to protect phenolic compounds from light exposure (Chedea et al, 2009).

HPLC-UV-DAD and HPLC-MS-ESI analysis

An Agilent 1100 LC-MSD System controlled by Agilent software v. A. 09.03 (Agilent Technologies, Waldbronn, Germany) equipped with a Vacuum degasser, a quaternary pump, an autosampler, a DAD variable wavelength detector 1100, 6-port autoinjector valve, was used for the HPLC-UV-DAD analysis. The HPLC analysis were performed using an octadecyl column, Phenomenex Luna C18 column, 100Å (4.6 mm i.d.× 250 mm; particle size 10µm), maintained at 35°C coupled with a Phenomenex C18 (ODS, Octadecyl) security guard column. Determination of phenolic compounds was carried out at 220, 280 and 320 nm. To prove the absence vs presence of anthocyans, reading was also performed at 520 nm (Chedea et al, 2009).

HPLC separation efficiency was optimized with different solvent ratios using different gradient conditions with water, methanol and acetonitrile, flow of 1ml/min and injection volume of 20µl. The mobile phase consisted of 95.0 % water (0.2% acetic acid), methanol (2.5%) and acetonitrile (2.5%). Gradient elution for the HPLC separation is explained in Tab. 1 (Chedea et al, 2009).

Tab. 1

Ternary mobile reversed phase gradient of the HPLC method (Chedea et al, 2009)

Time (min)	Flow rate (ml/min)	% of mobile phase A[a]	% of mobile phase B[b:c]
0.00	1.0	95.0	5.0
10.00	1.0	70.0	30.0
20.00	1.0	65.0	35.0
25.00	1.0	60.0	40.0
30.00	1.0	95.0	5.0
32.00	1.0	95.0	5.0

[a]Mobile phase A: a mixture of 0.2% acetic acid in water with pH= 3.1
[b]Mobile phase: acetonitrile
[c]Mobile phase: methanol
Mobile phase B was a 1:1 mixture of b:c

LC-MSD, Agilent 1100 mass spectrometry system (ESI), was used for detection and characterization of metabolites, using both negative and positive mode with a heated capillary at 350ºC and voltage of 4.1kV, nebulizer pressure 50.0 psi, a carrier gas flow (nitrogen) of 13 l/min. The full scan mass spectra of the phenolic compounds were measured from m/z 100 up to m/z 1000 (Chedea et al, 2009).

Quantification, repeatability, recovery

Quantification was performed using catechin as standards. A calibration curve was made over the range of 0.02 – 0.1 mg/ml (6 calibration points) of pure standard, diluted in HPLC grade methanol and the samples were analyzed in triplicate. The equation obtained for the catechin standard curve was y=12279x-38.679, r^2=0.99. Recovery through the SPE was calculated using o-cumaric acid as standard. In this case, the recovery was 102% (Chedea et al, 2009).

RESULTS AND DISCUSSION

Fig. 2 shows the LC-MS chromatogram of the LE extract recorded at 280 nm for the representative compounds of the extract together with the ionic MS fragmentation and UV-Vis spectra. The compounds were identified by retention time (t_R), UV spectrum (obtained from DAD) and MS spectrum (obtained from ESI detector). The structure of the reported compounds for the identified compounds has been tentatively assigned on the basis of previous citations in the literature (Klejdus et al, 2004; Heimler et al, 2004; Otieno and Shah, 2007; Deavours and Dixon, 2005).

The 3 isoflavones daidzein, glycitein, and genistein are each found in soy in 4 forms. In the soybean, the 6″-O-malonyl-β-glucosides of the isoflavones are the predominant forms. The 6″-O-acetyl-β-glucoside-, β-glucoside, and aglycone forms arise from degradation of the 6″-O-malonyl-β-glucosides during processing of soybeans and soy foods or during sample preparation and analysis (Collison, 2008). The fragmentation pattern of free genistein and daidzein malonylglucoside shows signals at m/z 269, and at m/z 501 and 253 respectively similar to those described by Heimler et al., 2004.

The identified compounds were tentatively identified as being 6″-O-malonyl-β-glucoside of daidzein (t_R=28,16) and the agluconic genistein (t_R=34,81), isoflavones known to be important bioactive compounds in soy beans. The UV-Vis absorption maxima for the spectra of malonyl daidzin were at 248 nm and 308 nm and for genistein at 260 nm.

The quantitative analysis based on spectrophotometric determination (UV-DAD) gave 4 mg/kg aliquots for malonyl daidzin and 10 mg/kg aliquots for genistein.

CONCLUSIONS

The analysed LE contains the glucosilated form of daidzein- the malonyl daidzin and free genistein but the amounts in which these are present are very low.

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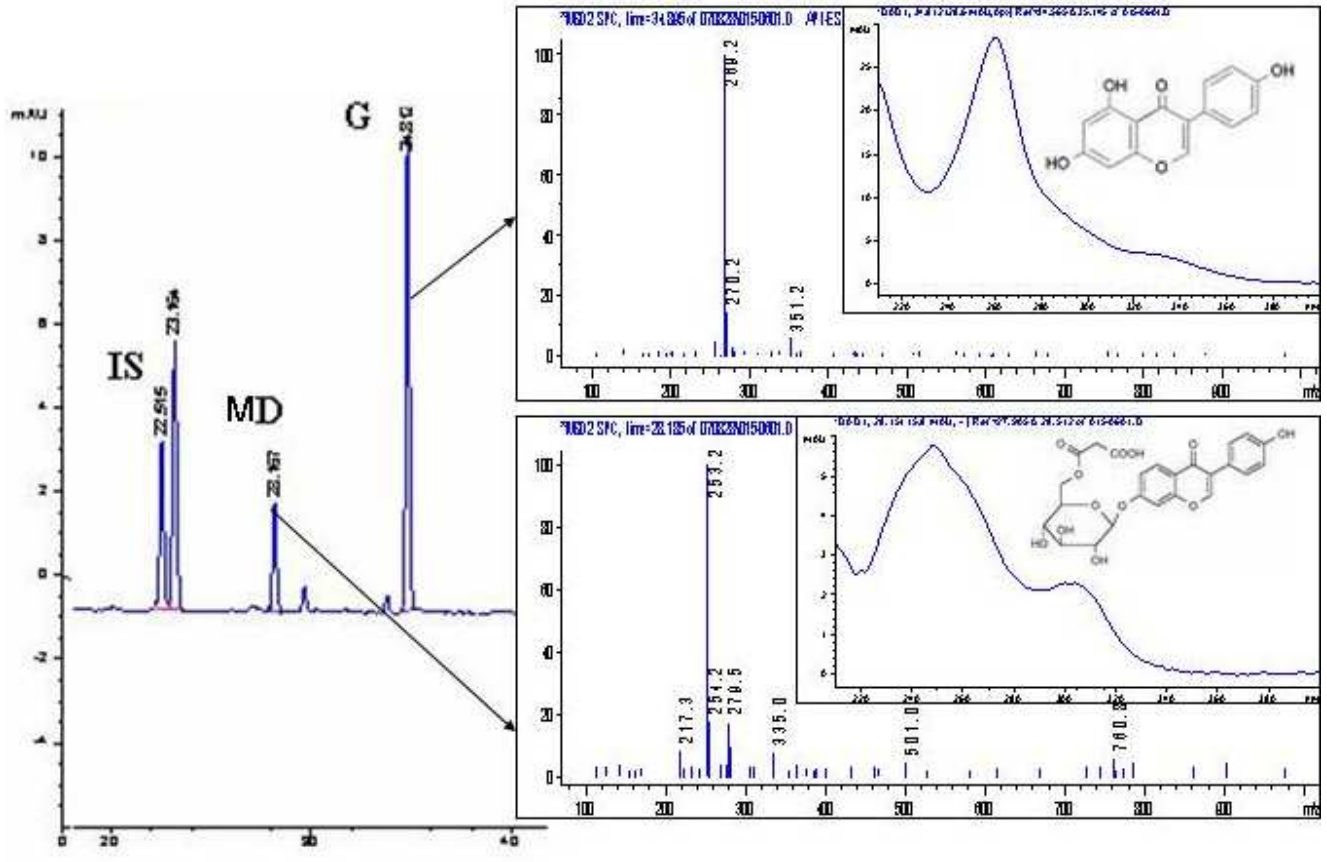

Fig. 2. LE chromatogram at 280 nm obtained by LC-MS analysis, showing the presence of the most important compounds, 6″-O-malonyl-β-glucoside daidzein (MD) and genistein (G). There are also presented the m/z ratio for the base peak $[M-H]^-$ from the MS spectra, the UV-Vis spectra and the chemical structures.

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