NPC Natural Product Communications

2010
Vol. 5
No. 11
1775 - 1780

HPLC/DAD/MS and Antioxidant Activity of Isoflavone-Based Food Supplements

Annalisa Romani[a], Pamela Vignolini [a*], Annalisa Tanini[b], Barbara Pampaloni[b] and Daniela Heimler[c]

[a]*Dipartimento di Scienze Farmaceutiche, Università di Firenze, via U. Schiff, 6, 50019 Sesto Fiorentino (FI), Italy*

[b]*Dipartimento di Medicina Intera, Università di Firenze, viale Pieraccini, 18, 50139, Firenze, Italy*

[c]*Dipartimento di Scienze delle Produzioni Vegetali, del Suolo e dell'Ambiente Agroforestale, Università di Firenze, P.le delle Cascine 18, 50144 Firenze, Italy*

pamela.vignolini@unifi.it

Received: June 1st, 2010; Accepted: July 20th, 2010

Isoflavones are polyphenolic compounds found mainly in legumes the benefits of which have been widely studied and attributed in particular to their phytoestrogenic activity. The aim of this study was to evaluate the quali-quantitative composition of food supplements based on soy isoflavones (*Glycine max* L.) and red clover (*Trifolium pratense*). Six commercial food supplements (five soy-based and one red clover-based) were analyzed by HPLC/DAD/MS. Genistein, daidzein, glycitein, biochanin A and formononetin derivatives (glycosides and acylglycosides) were identified in the analyzed samples. Also the antiradical activities (towards the DPPH• radical) and Fe^{2+} chelating abilities were compared.

Keywords: Antiradical activity, soy extracts, total phenolics, iron ion chelating ability, quantitative analysis.

Isoflavones are widely distributed in Leguminosae species. These substances are structurally similar to the mammalian estrogen estradiol-17β and exhibit estrogenicity. These compounds exhibit broad-spectrum antimicrobial activity and, therefore, their role in plants is mainly linked to fighting microbial diseases [1].

Phytoestrogens have received much attention due to their importance in the diet of Oriental people, who are less affected by so-called Western diseases which include hormone-dependent tumors, osteoporosis and cardiovascular disorders [2]. The major sources of phytoestrogens in human diets are isoflavones in soybeans and soy foods; isoflavones are also found in chick peas, beans, peas, and groundnuts [3]. Recent research has shown also that red clover (*Trifolium pratense* L.) contains isoflavones [4]. However, in the Western diet, food supplements play an important role in the isoflavone balance. The isoflavone composition and antiradical characteristics in diet food supplements are strictly related to the vegetal material used in their formulation. These commercial supplements generally contain extracts from soy, red clover and kudzu plants, which exhibit different isoflavone profiles.

Soy seeds contain mainly genistein, daidzein and glycitein derivatives [5]; red clover mainly genistein, daidzein, glycitein, biochanin A, pratensein, and formonetin derivatives [6]; and kudzu mainly puerarin, daidzein and genistein derivatives [7].

The health benefit of a food supplement can be linked also to its antiradical activity. Free radicals are generated in small amounts by normal processes of metabolism, but free radicals cause the oxidation of biomolecules (proteins, amino acids, lipids, and DNA), which leads to cell injury [8]. The principal defense systems against oxygen free radicals are enzymatic pathways (for example, SOD, GSH peroxidases, and glutathione reductase) and antioxidant food. With regard to antioxidant properties, isoflavones are not included among the most efficient antioxidant flavonoids [9], but isoflavones are superior in inhibiting LDL oxidation induced by copper ions [10]. Since the protective effects of flavonoids are ascribed to their ability to transfer electrons from free radicals, chelate metals, reduce alpha-tocopherol radicals and inhibit oxidases [11], the antiradical activity can be accessed through different assays.

The separation and identification techniques of phytoestrogenic compounds have recently been reviewed [12], and the most used technique is HPLC coupled with either UV or MS detection.

Regarding antioxidant capacity, there are critical reviews on its significance and characteristics [13, 14]. The methods can be divided into two main groups that differ for the chemical reactions which are taken into account, i.e. hydrogen atom transfer (HAT assays) and single electron transfer (SET assays) [15]. This is a rough classification since it does not take into account assays which may involve both kinds of reactions and assays which measure other radical oxygen species (ROS) [15]. All this shows how the simple definition of "antioxidant capacity" is not at all simple. Our choice was the use of a typical SET assay, the DPPH• test, which is mainly regarded as a SET assay since the DPPH• radical reacts with an oxidant species (ROS), transferring an electron, but it can also be neutralized via H-abstraction reactions [16]. The phenolic content is generally determined through the Folin-Ciocalteu assay; this assay, however, involves a redox reaction and can, therefore, be associated with the antioxidant capacity of the sample. Transition metals have a role in the generation of oxygen free radicals through Fenton type reactions; since polyphenols may chelate Fe^{2+} ions, the evaluation of the Fe(II) ion binding property of polyphenols can focus on a different way of acting as an antioxidant substrate.

Commercial dietary supplements were analyzed for their antioxidant activity [17], and their isoflavone content was determined by means of HPLC [18-20].

In order to assess the different properties of isoflavone-based food supplements and to verify that the content of the tablets was in agreement with what is declared by the producer, we compared the HPLC/DAD/MS analysis with the results of rapid tests so that a better understanding of the alimentary properties of food supplements could be achieved and information about which tests, other than the widely-used and time-consuming HPLC/DAD/MS analysis, could be used to evaluate the final quality of the commercial product.

The characteristics, as taken from the labels of the commercial isoflavone-based supplements, are reported in Table 1; only one supplement contained clover isoflavones, while all the others were soy-based.

The chromatographic profile of a food supplement sample (FS4), using the "long" method, which has already been used successfully for the separation of isoflavones [5], identified compounds including isoflavone aglycones (daidzein, genistein and glycitein) and isoflavone derivatives. Since the overall time of analysis was quite long, we compared this method with a shorter one already used for soybean seeds [21]. The chromatogram obtained with the "short" method, described in the experimental section, detected all the compounds previously identified with the longer analysis procedure. The shorter method offers improvements with respect to the longer one, for example, the same resolution as the longer method was obtained, with correct quantitative analysis, and with savings in terms of solvents. In order to obtain further solvent savings we used a shorter column with a low flow rate (0.2 mL min^{-1}); in this case we also found the previously identified compounds, and obtained the same resolution as that of the longer column.

Table 1: Commercial food supplements, as described on their labels.

Food-supplement	Content
FS1	Soy isoflavones Vitamins C, E, B6, B2, B1, D. Calcium, Phosphorus
FS2	Dry extract of *Trifolium pratensis*
FS3 1	soy isoflavones, *Equisetum arvense* extract
FS4	Soy isoflavones
FS5	Soy isoflavones
FS6	Soy isoflavones, *Griffonia simplicifolia* seeds, *Cimicifuga racemosa* rhizome

The extraction yield (95%) was controlled by adding gallic acid as an internal standard. The choice of this molecule was based on its absence in our samples, and because of its retention time, which falls in an empty zone of the chromatogram (RT = 2.75 min for the "long" method with a Nova Pak C18 column, and RT=1.98 min for the shorter one with a Luna C18 column).

Table 2 reports the amounts of the different isoflavones in the six samples. The qualitative composition of FS2 is different since it is a clover-based supplement. However, even in the case of the soy-based supplements, there are some differences in the amounts of single isoflavones. In particular, genistein and daidzein glucoside are the main compounds and they ranged from 43.3% to 62.9% and from 17% to 35.5 %, respectively. It should be noted that in addition to FS2, FS6 also contained biochanin.

Table 3 lists the total amounts of isoflavones from HPLC data compared with the amounts reported on the labels of the commercial products; the data agree quite well.

As regards the antioxidant and antiradical activity, we considered the data of three tests: the Folin test, which, especially in the case of commercial samples [21], can be related to the antioxidant activity of the commercial product more than to its polyphenol content; the DPPH

Table 2: Amount of different isoflavones (mg/g) from HPLC data.

Com-pounds	r.t	FS1	FS2	FS3	FS4	FS5	FS6
G der	11.10	0.07	-	0.07	0.06	0.07	0.12
Gly der	11.74	-	-	0.09	-	-	-
G der	12.17	0.47	-	-	-	-	-
Gly der	13.30	-	-	0.42	0.11	0.16	0.32
D 7-O-gluc	13.59	22.15	-	26.55	9.18	13.09	26.79
Gly gluc	14.11	2.21	-	9.41	0.57	0.94	3.22
G 7-O-gluc	16.07	43.84	-	34.57	32.01	35.78	39.64
D der	16.11	-	-	-	Traces	Traces	-
D aclgluc	18.79	-	-	1.72	0.25	0.56	1.40
Gly aclgluc	19.05	-	-	0.55	-	-	0.12
G der	19.26	0.07	-	-	1.61	1.19	-
D der	19.52	-	0.81	-	-	-	-
D	20.41	0.34	0.25	3.30	4.21	3.77	1.23
Gly	20.91	0.12		1.57	0.78	1.21	1.67
G der	21.19	-	0.66	-	-	-	-
G der	21.89	-	0.39	-	-	-	-
G der	22.77	-	0.85	-	-	-	-
G	22.83	0.34	0.28	1.57	4.27	4.11	0.67
G der	23.36	-	0.90	-	-	-	-
F	24.36	-	21.93	-	-	-	-
D der	24.53	0.02	-	Traces	-	-	-
G der	25.64	-	1.33	-	-	-	-
B	26.16	-	19.12	-	-	-	0.19

D = daidzein, Gly = glycitein, G = genistein, F = formonetin, B = biochanin, gluc = glucoside, acgluc = acetylglucoside
Data are the mean of three determinations (standard deviation < 3%

Table 3: Amounts of isoflavones (g/100g) from HPLC data and as written on the labels of the commercial products.

sample	Isoflavones HPLC	Isoflavones labels
FS1	6.96	7.41
FS2	4.57	4
FS3	7.98	6.66
FS4	5.3	5.59
FS5	6.08	5.59
FS6	7.53	6.52

Table 4: Data from the Folin test expressed as g gallic acid/ 100 g sample; DPPH·data expressed as IC_{50} mg sample / mg DPPH·; Fe(II) chelating ability expressed as mg sample that halves the absorbance of ferrozine according to the experimental protocol.

sample	Folin	DPPH·	Fe(II) chelating ability
FS1	3.34	6.19	0.48
FS2	2.96	49.86	0.50
FS3	1.44	28.42	0.75
FS4	1.25	77.01	0.25
FS5	1.51	71.12	0.23
FS6	3.71	0.87	0.98

test, which accounts for the antiradical activity of the sample; and the iron (II) chelating ability. The data are reported in Table 4. The data from the Folin-Ciocalteu analysis in this case are not correlated at all with the isoflavone content. This method is affected by a large number of interfering compounds [14] and isoflavones, on the other hand, do not react very much with this reagent. In fact, the highest values were obtained in the case of FS1 (which contains vitamin C) and FS6 (which contains *Griffonia simplicifolia* seeds and *Cimicifuga racemosa* rhizome). It should also be noted that the data are expressed as gallic acid content, which has a lower molecular weight than genistein (170 compared to 270).

In the case of the DPPH· test there is a good correlation (R^2 = 0.9785) between antiradical activity and daidzein content (under all its forms) if FS1 (containing vitamin C) and FS6 (see above) are not considered. Even in these cases, however, the total composition of the food supplements affects the results, since Lee *et al.* [22] found similar free radical scavenging activity for genistein and daidzein, and genistin activity higher than that of daidzin in a study on pure isoflavones separated from dried soybeans.

The Fe(II) chelating ability correlates quite well (R^2 = 0.8415) in all samples with their daidzein content.

From these data some conclusions on the analysis of these kinds of food supplements can be drawn. On the labels of commercial products only the isoflavone content is reported, however some characteristics (e.g. antioxidant and antiradical activity) are related to the presence of other components, which may change the biological activity of the supplement. Therefore, an accurate HPLC/DAD/MS method, together with rapid spectrophotometric tests, may describe better the specific characteristics of each supplement. It should also be noted that, in the case of food supplements with similar composition, there is a direct proportionality between HPLC results and some rapid tests.

Epidemiological studies suggest that consumption of isoflavones rich diets can improve several postmenopausal complications. Pampaloni *et al.* [23] have investigated the absorption and efficacy of isoflavonic supplementation in the treatment of menopausal symptoms using the commercial isoflavone-based supplements reported in Table 1. They showed that administration of isoflavone supplements produced a decrease of symptoms in this cohort of postmenopausal women monitored for isoflavone absorption.

Experimental

Food supplements: Six commercial, soy-based food supplements, representative of the Italian markets, were analyzed. These were provided by the gynecology outpatient Clinic of the Bone Metabolic Unit of the University of Florence.

Standards and solvents: Authentic standards of daidzein, genistein and gallic acid were purchased from Extrasynthèse S.A. (Lyon, Nord-Genay, France). All the solvents used were of HPLC grade purity (BDH Laboratory supplies, England).

Sample preparation: A 250 mg of sample flour was extracted over night at room temperature with 25 mL of

ethanol/water (70/30, v/v, made to pH 3.2 with HCOOH). The solution was filtered before HPLC analysis and 2 μL (20 μg) of the extract was injected.

HPLC/DAD analysis: The analyses were carried out using a HP 1100L liquid chromatograph equipped with a DAD detector (Agilent Technologies, California, USA). Polyphenolic compounds were separated using a 150 × 3.9 mm (4 μm) Nova Pak C18 column (Waters Corporation, Massachusetts USA), and a 50 × 2.2 mm (3 μm) Luna C18 column (Phenomenex), operating at 26°C. UV/Vis spectra were recorded in the 190-450 nm range and the chromatograms acquired at 260, 305, 330 and 350 nm. The mobile phase was a four-step linear solvent gradient system, starting from 95% H_2O (adjusted to pH 3.2 with HCOOH) up to 100% CH_3CN during a 27 min period [21], and a four-step linear solvent gradient system, starting from 91% H_2O (adjusted to pH 3.2 with HCOOH) up to 100% CH_3CN during an 18 min period [21].

HPLC/MS analysis: HPLC/MS analyses were performed using a HP 1100 MSD API, ESI interface, coupled with a HP 1100L liquid chromatograph equipped with a DAD detector (Agilent Technologies). The HPLC/MS analysis was performed using the same HPLC/DAD condition with water adjusted to pH 3.2 with HCOOH. Mass spectrometer operating conditions were: nitrogen gas temperature 350°C at a flow rate of 10.5 L min^{-1}, nebulizer pressure 30 psi, quadrupole temperature 30°C, and capillary voltage 3500 V. The mass spectrometer was operated in positive and negative mode at 80-180 eV fragmentor values.

Identification and quantification of individual isoflavones: Identification of individual isoflavones was carried out using their retention times and both spectroscopic and spectrometric data. Individual isoflavones were quantified by a four-point regression curve ($r^2 \geq 0.9998$) operating in the range 0-10 μg on the basis of authentic standards, and determination was directly performed by HPLC/DAD. The detection limits, evaluated for genistein and daidzein in all analyzed extracts, are 200 μg kg^{-1} [21]. In particular, genistein, glycitein and biocanin A derivatives were determined at 260 nm using genistein as reference compound, while daidzein and formononetin derivatives were determined at 305 nm using daidzein as reference compound. In all cases, actual concentrations of the derivatives were calculated after applying corrections for differences in molecular weight.

Antiradical activity: Free radical scavenging activity was evaluated with the DPPH• (1,1-diphenyl-2-picrylhydrazyl radical) assay. The antiradical capacity of the sample extracts was estimated according to the procedure reported by Brand-Williams [24] and slightly modified. Two mL of the sample solution, suitably diluted with ethanol, was added to 2 mL of an ethanol solution of DPPH• (0.0025g/100mL) and the mixture kept at room temperature. After 20 min, the absorption was measured at 517 nm with a Lambda 25 spectrophotometer (Perkin-Elmer) versus ethanol as a blank. Each day, the absorption of the DPPH• solution was checked. The antiradical activity is expressed as IC_{50}, the antiradical dose required to cause a 50% inhibition. IC_{50} was calculated plotting the ratio: $(A_{blank}-A_{sample}/A_{blank}) \; x \; 100$, where A_{blank} is the absorption of the DPPH• solution and A_{sample} is the absorption of the DPPH• solution after addition of the sample, against the concentration of the sample. IC_{50} is expressed as mg sample /mg DPPH•.

Total phenolic content: The total phenolic content was determined using the Folin-Ciocalteu method, described by Singleton *et al*. [25] and slightly modified according to Dewanto *et al*. [26]. To 125 μL of the suitably diluted sample extract, 0.5 mL of deionized water and 125 μL of the Folin-Ciocalteu reagent were added. The mixture was kept for 6 min and then 1.25 mL of a 7% aqueous Na_2CO_3 solution was added. The final volume was adjusted to 3 mL with water. After 90 min, the absorption was measured at 760 nm against water as a blank. The amount of total phenolics is expressed as gallic acid equivalents (GAE, mg gallic acid/100 g sample) through the calibration curve of gallic acid. The calibration curve ranged from 20 to 500 μg/mL (R^2 = 0.9969).

Fe^{2+} chelating ability: The chelating activity of samples on Fe^{2+} was measured according to Duh *et al.* [27] and the original work of Stookey [28] with modifications. The desired volume of plant extract was diluted to 1.5 mL with 0.25 M acetate buffer (pH 4.75) then 25 μL of 2 mM $FeCl_2$ and 1 mL of the same solvent in which the plant extracts or standards were dissolved were added. The solution was incubated at room temperature for 20 min. After incubation, 100 μL of 5 mM ferrozine were added, the mixture was shaken and the absorbance was measured after 5 min at 562 nm versus ethanol/water (70/30, v/v). The ability of chelating ferrous ions was expressed as the amount of sample (mg) that halves the absorbance of the control (all reagents without the sample), calculated plotting the ratio $(A_{control}-A_{sample}/A_{control}) \; x \; 100$ against the concentration of the sample.

Acknowledgments - The research was supported by funds from Provincia di Grosseto (Italy) and Ente Cassa di Risparmio of Florence.

References

[1] Dixon RA, Ferreira D. (**2002**) Genistein. *Phytochemistry*, ***60***, 205-211.

[2] Adlercreutz H. (**1990)** Western diet and Western diseases: some hormonal and biochemical mechanism and associations. *Scandinavian Journal of Clinical. Laboratory Investigation Supplementum*, ***201***, 3-23.

[3] Mazur WM, Duke JA, Wahala K, Rasku S, Adlercreutz H. (**1998**) Isoflavonoids and lignans in legumes: nutritional and health aspects in humans. *Journal of Nutritional Biochemistry*, ***9***, 193-200.

[4] Dornstauder E, Jisa E, Unterrieder I, Krenn L, Kubelka W, Jungbauer A. (**2001**) Estrogenic activity of two standardized red clover extracts (MenoflavonReg) intended for large scale use in hormone replace therapy. J*ournal of Steroid Biochemistry and Molecular Biology*, ***78***, 67-75

[5] Romani A, Vignolini P, Galardi C, Aroldi C, Vazzana C, Heimler D. (**2003**) Polyphenolic content in different plant parts of soy cultivars grown under natural conditions. *Journal of Agricultural and Food Chemistry*, ***51***, 5302-5306.

[6] Tsao R, Papadopoulos Y, Yang R, Young JC, McRae K. (**2006**) Isoflavone profiles of red clover and their distribution in different parts harvested at different growing stages. *Journal of Agricultural and Food Chemistry*, ***54***, 5797-5805.

[7] Prasain JK, Jones K, Kirk M, Wilson L, Smith-Johnson M, Weaver C, Barnes S. (**2003)** Profiling and quantification of isoflavonoids in kudzu dietary supplements by high performance liquid chromatography and electrospray ionization tandem mass spectrometry. *Journal of Agricultural and Food Chemistry*, ***51***, 4213-4218.

[8] Fang YZ, Yang S, Wu G. (**2002**) Free radicals, antioxidants, and nutrition. *Nutrition*, ***18***, 872-879.

[9] Rice-Evans CA, Miller NJ, Paganga G. (**1997**) Antioxidant properties of phenolic compounds. *Trends in Plant Science***, *2***, 152-159.

[10] Vaya J, Mahmood S, Goldblum A, Aviram M, Volkova N, Shaalan A, Musa R, Tamir S. (**2003**) Inhibition of LDL oxidation by flavonoids in relation to their structure and calculated enthalpy. *Phytochemistry*, ***62***, 89-99.

[11] Heim KE, Tagliaferro AR, Bobilya DJ. (**2002**) Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *Journal of Nutritional Biochemistry*, ***13***, 572-584.

[12] Wu Q, Wang M, Simon JE. (**2004**) Analytical methods to determine phytoestrogenic compounds. *Journal of Chromatography B*, ***812***, 325-355.

[13] Prior RL, Cao G. (**2000**) Analysis of botanicals and dietary supplements for antioxidant capacity: a review. *Journal of AOAC International*, ***83***, 950-956.

[14] Prior RL, Wu X, Schaich K. (**2005**) Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, ***53***, 4290-4302.

[15] Huang D, Ou B, Prior RL (**2005**) The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*, ***53***, 1841-1856.

[16] Jiménez A Selga A, Torres JL, Julià L. (**2004**) Reducing activity of polyphenols with stable radicals of the TTM series electron transfer versus H-abstraction reactions in flavan-3-ols. *Organic Letters*, ***6***, 4583-4586.

[17] Davalos A, Gomez-Cordovès C, Bartolomé B. (**2003**) Commercial dietary supplements assayed for their antioxidant activity by different methodologies *Journal of Agricultural and Food Chemistry*, ***51***, 2512-2519.

[18] Chen LJ, Zhao X, Plummer S, Tang J, Games DE. (**2005**) Quantitative determination and structural characterization of isoflavones in nutrition supplements by liquid chromatography – mass spectrometry. *Journal of Chromatography A*, ***1082***, 60-70.

[19] Delmonte P, Perry J, Rader JI. (**2006**) Determination of isoflavones in dietary supplements containing soy, red clover and kudzu: extraction followed by basic or acid hydrolysis. *Journal of Chromatography A*, ***1107***, 59-69.

[20] Griffith AP, Collins MW. (**2001**) Improved methods for the extraction and analysis of isoflavones from soy-containing foods and nutritional supplements by reversed-phase high-performance liquid chromatography and liquid chromatography – mass spectrometry. *Journal of Chromatography A*, ***913***, 397-413.

[21] Heimler D, Vignolini P, Galardi C, Pinelli, P, Romani A. (**2004**) Simple extraction and rapid quantitative analysis of isoflavones in soybean seeds. *Chromatographia***, *59***, 361-365.

[22] Lee CH, Yang L, Xu JZ, Yeung SYV, Huang Y, Chen ZY. (**2005**) Evaluation of estrogenic activity of plant extracts for the potential treatment of menopausal symptoms. *Journal of Agricultural and Food Chemistry*, ***90***, 735-741.

[23] Pampaloni B, Bartolini S, Bartolini E, Ottanelli O, Masi L, Romani R, Tanini A, Vignolini P, Brandi ML. (**2009**) Absorption and bioeffects of an isoflavone-based supplementation in postmenopausal women. *Clinical Cases in Mineral and Bone Metab*olism Sep–Dec. ***6***, 254–260.

[24] Brand-Williams W, Cuvelier ME. (**1995**) Use of a free radical method to evaluate the antioxidant activity. *LWT. Food Science and Technology*, ***28***, 25-30.

[25] Singleton VL, Orthofer R, Lamuela-Raventos RM. (**1999**) Analysis of total phenols and other oxidation substrates and antioxidants by means of the Folin-Ciocalteu reagent. *Methods in Enzymology*, ***299***, 152-178.

[26] Dewanto V, Wu X, Adom KK, Liu RH. (**2002**) Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *Journal of Agricultural and Food Chemistry*, ***50***, 3010-3014.

[27] Duh PD, Yen GC, Yen WJ, Chang LW. (**2001**) Antioxidant effects of water extracts from barley (*Hordeum vulgaris*) prepared under different roasting temperature. *Journal of Agricultural and Food Chemistry*, ***49***, 1455-1463.

[28] Stookey LL. (**1970**) Ferrozine: a new spctrophotometric reagent for iron. *Analytical Chemistry*, ***42***, 779-781