ASSESSMENT OF ISOFLAVONE AGLYCONES VARIABILITY IN SOY FOOD SUPPLEMENTS USING A VALIDATED HPLC-UV METHOD

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Abstract

Background and aims. Soy supplements are often recommended in the management of menopause symptoms. The declared content of soy supplements is commonly expressed as total isoflavones per dosage form. Given that soy isoflavones have different estrogenic potencies, pharmacokinetics and metabolism, the aim of this study was to evaluate the total isoflavone content and the aglycone profile of seven soy supplements and one soy seed extract. Label accuracy was assessed, in relation to the precise content and the recommended posology for estimating whether the optimal dose is achieved for alleviating menopause symptoms.

Methods. A high performance liquid chromatography method was developed for evaluating the aglycone content (genistein, daidzein, glycitein). After extraction and acidic hydrolysis, the aglycones were separated on a C18 column, using 0.1% acetic acid and acetonitrile as mobile phases. The flow rate was 1.5mL min\(^{-1}\) and the UV detector wavelength was set at 260nm. A linear relationship was found in the range 5-80µg mL\(^{-1}\). The method was validated using the accuracy profile methodology.

Results. The total isoflavone content ranged from 6.07 to 41.68mg dosage form\(^{-1}\). Various aglycone profiles were obtained for each supplement which can result in a different estrogenic activity, bioavailability and finally, in a different efficiency in alleviating menopause symptoms. In most clinical trials where soy isoflavones were evaluated, little attention was paid to determining the exact aglycone profile of the employed soy extracts.

Conclusions. As clinical outcomes continue to be controversial, this study highlights the need of standardization in genistein, rather than total isoflavones and labeling accuracy for soy supplements.

Keywords: food supplement, genistein, HPLC, isoflavones, standardization

American and European countries. Approximately 10–25% of Chinese women and 10–20% of Indonesian women report vasomotor symptoms compared with 60–90% of North American and European women. These findings are alleged to be, at least in part, related to soy-rich diets [1]. Soy food is an integrant part of the traditional Asian...
diet, daily intake of soy protein being estimated at 20-30g. Conversely, a non-Asian diet contains less than 1g of soy protein day\(^{-1}\) [2]. Therefore, in order to benefit from soy intake, non-Asian women are turning to soy based supplements, a rapidly growing segment of pharmaceutical market.

These supplements contain soy extracts with different amounts of isoflavones. Isoflavones are a class of polyphenolic compounds with weak estrogenic proprieties that have been promoted as natural remedies in relieving menopausal symptoms, decreasing the risk of cardiovascular disease and osteoporosis, as well as protecting against several forms of cancer [3]. The main soy isoflavones are daidzein (D), glycitein (GY) and genistein (GN), presented as glycosides or as esterified glycosides and, in a minor degree, as free forms also known as aglycones (Figure 1).

**Figure 1.** Chemical structure of soybean isoflavones.

| Aglycones   | R₁ | R₂ | R₃ |
|-------------|----|----|----|
| Daidzein    | H  | H  | H  |
| Genistein   | OH | H  | H  |
| Glycitein   | H  | OCH₃| H  |

**Conjugated forms**

| Aglycones | R₁ | R₂ | R₃ |
|-----------|----|----|----|
| Daidzein  | H  | H  | CH₂O₃|
| Genistein | OH | H  | CH₂O₃|
| Glycitein | H  | OCH₃| CH₂O₃|

For each supplement, the average weight was calculated and the samples were pulverized into a fine powder to ensure a homogenous mixture.

According to their labels, the soy supplements contained also low doses of vitamins, minerals or other isoflavone free botanicals, like *Hypericum perforatum* or *Terminalia arjuna* (Table I). Supplements containing also “kudzu” root (*Pueraria radix*) or clover (*Trifolium pretense L*) were excluded since our study was focused only on soy isoflavones.

For each supplement, the average weight was calculated and the samples were stored at room temperature, protected from light, until analysis.

A sample portion of 5 to 155 mg was accurately weighed into 10 mL volumetric flasks and 5mL of 3M HCl methanolic solution was added. To achieve quantitative extraction, the solution was continuously shaken at 500 rpm for 30 minutes using a laboratory vortex-mixer (Multi-Pulse Vortexer, Glas-Col®, USA). After extraction, the volume was adjusted to 10 mL with the same solvent.

For the soy seed extract, 20 µL extraction solvent was directly injected into the HPLC system, without previous hydrolysis.

The acidic hydrolysis was performed for the isoflavones extracts obtained from soy seed extract and symptoms is achieved.

**Materials and methods**

**Chemicals, standards and stock solutions**

Acetonitrile was purchased from Merck Millipore (Darmstadt, Germany) and methanol, dimethylsulfoxide, acetic acid and hydrochloric acid were obtained from Sigma-Aldrich Chemical Co. (St. Louis, Mo., USA). Ultrapure water was prepared using a Milli-Q System (Millipore S.A. Molshein, France). All chemicals were HPLC grade or equivalent.

The standards for isoflavone aglycones, genistein, daidzein and glycitein, all of >99% purity, were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo., USA).

Stock solutions were prepared by dissolving accurately weighted amounts of GN and D in methanol and only GY was dissolved in dimethylsulfoxide due to its higher hydrophobicity. The obtained stock solutions (1mg mL\(^{-1}\)) were stored in sealed 2 mL vials at -20°C, protected from light.

**Collection and preparation of samples**

Seven over-the-counter soy based supplements (S1-S7) were purchased from local pharmacies in Cluj-Napoca, Romania, in May 2013, in intact retail packaging and within validity period.

According to their labels, the soy supplements contained also low doses of vitamins, minerals or other isoflavone free botanicals, like *Hypericum perforatum* or *Terminalia arjuna* (Table I). Supplements containing also “kudzu” root (*Pueraria radix*) or clover (*Trifolium pretense L*) were excluded since our study was focused only on soy isoflavones.

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The acidic hydrolysis was performed for the isoflavones extracts obtained from soy seed extract and
soy supplements. The optimal conditions for maximal conversion rate to aglycones were chosen based on previous studies [6]. Briefly, 0.50 mL of each extraction solvent was transferred to a microcentrifuge tube and heated for 120 minutes at 80°C in a digital dry bath (Bio-Rad Laboratories, USA) for the complete hydrolysis of the glycosidic forms. After cooling at room temperature, the hydrolyzed extract was diluted to 1mL with methanol. Immediately after hydrolysis, the samples were analyzed by high performance liquid chromatography (HPLC).

**Chromatographic separation and quantification**

The HPLC analysis was performed with a 2695 Alliance Chromatographic System, equipped with quaternary pumps, an in-line degasser, a thermostatic autosampler and a 966 Photodiode Array Detector (Waters Corp., Milford, MA, USA). Separation of isoflavone aglycones was accomplished using a 4.6x150 mm i.d., 5 µm particle size dC18 Atlantis column (Waters Corp., Milford, MA, USA). Twenty microliters of standard solutions or sample hydrolysate, were respectively injected into the column and eluted at a flow rate of 1.5 mL min⁻¹. The binary mobile phase consisted of 0.1% acetic acid in water (solvent A) and 0.1% acetic acid in acetonitrile (solvent B).

The gradient program was set as follows: 0-12min from 12% to 30% solvent B; 12-18min from 30% to 60% solvent B; 18-19min from 60% to 12% solvent B; 19-28min, re-equilibration with 12% solvent B. For peak identification, on-line UV spectra were recorded in the range of 190-400 nm and absorbance at 260 nm was used for quantification. Data acquisition was carried out using Empower 2.0 Software (Waters Corp., Milford, MA, USA). Isoflavone aglycones were identified based on retention times and confirmed by UV spectra. For the quantification assay, the system was calibrated for D, GY and GN with a five point regression curve in a range of 5-80 µg mL⁻¹ for all aglycones.

As previous studies have shown [6], the stability of genistin in acidic conditions is limited (<4h). Thus, it is important to perform the quantification in a short period of time. In our case, the isoflavone content of soy food supplements was determined in a total run time of 28 minutes, including the column re-equilibration after gradient elution. In this way, we ensured the entire stability of genistin.

**Validation procedure: accuracy profile**

The validation design consists of three days, three concentration levels and three repetitions. Altogether, the number of validation trials was 27. In order to carry out the validation procedure, two sets of standards were prepared: calibration standards and validation standards.

Validation standards were obtained by spiking a blank matrix of excipients with the amount of soy seed extract equivalent to 50mg isoflavones expressed as aglycone equivalents. All the validation standard solutions have been prepared by independent weighing of soy seed extract within matrix at three concentration levels: low, medium and high. These concentrations have been selected after measuring the exact amount of aglycones from the soy seed extract and cover the variations in amounts of aglycones in every analyzed sample.

Calibration standards were prepared by independent weighing of the soy seed extract, without matrix, at middle and high concentration levels.

The statistical analysis was performed using Microsoft Excel TM 2010 and for graphic illustration we used Metlab R2008b software.
Results

Method validation

Peak areas were directly proportional to concentrations for all isoflavone aglycones, with correlation coefficients >0.999. After processing experimental data according to accuracy profile method validation, the performance criteria were determined for each level of concentration (Table II). Most of these criteria are expressed both in absolute and relative value.

As illustrated in Figure 2, the relative β-expectation tolerance limits did not exceed the acceptance limits of ±20% showing that the method is valid for the quantification of all aglycones. The predicted concentrations were close to the introduced concentrations, with absolute bias ranging from -0.33% to 0.11% and relative bias ranging from -0.79% to 0.61%.

Table II. Validation results obtained for all aglycones.

| Validation criteria          | Daidzein  | Glycitein | Genistein |
|-----------------------------|-----------|-----------|-----------|
| Mean introduced concentration | 24.37     | 10.96     | 4.59      |
| Lower β tolerance limit     | 21.15     | 9.12      | 3.86      |
| Upper β tolerance limit     | 27.59     | 12.80     | 5.32      |
| Lower relative β tolerance limit (%) | -13.20   | -16.49    | -15.77    |
| Upper relative β tolerance limit (%) | -4.96    | -11.12    | -13.10    |
| Repeatability standard deviation | 1.09     | 0.48      | 0.29      |
| Intermediate precision standard deviation | 1.23     | 0.61      | 0.29      |
| Repeatability RSD (%)      | 4.48      | 4.33      | 6.36      |
| Intermediate precision RSD (%) | 5.03      | 4.37      | 4.79      |
| Predicted concentration    | 23.94     | 10.77     | 4.51      |
| Absolute bias (%)          | 0.00      | 0.03      | 0.00      |
| Relative bias (%)          | 0.01      | 0.24      | 0.06      |
| Recovery (%)               | 100.01    | 100.24    | 100.06    |

Figure 2. Accuracy profiles obtained for the quantification of (a) daidzein, (b) glycitein and (c) genistein; plain line: relative bias, dashed line: β-expectation tolerance limits, dashed-dotted line: acceptance limit (%).
isoﬂavone proﬁle and content in food supplements and soy seed extract

The soy seed extract was analyzed before and after acidic hydrolysis. Hydrolysis efficiency was evaluated by monitoring the production of aglycones and disappearance of their glycosidic forms in soy extract. Using 3.0 M HCl methanolic solution, the aglycones reached the highest content and peaks corresponding to the glycosides were not detected (Figure 3, Ea and Eb).

For the soy extract, the total amount of aglycones was 27.05 mg 100 g extract\(^{-1}\), while for the soy food supplements, the total concentration of isoﬂavone aglycones ranged from 3.48 to 23.99 mg dosage form\(^{-1}\) (Table III).

As none of the manufacturers labeled the ratio of aglycones to glycoside forms or their individual concentrations, the declared amount comprise, most likely, the concentration of all soy isoflavones (the weight of free and conjugated forms). Therefore, in order to estimate the total isoﬂavone content, it is necessary to take into account the proportion of free and conjugated forms. The ratio 1:52:9:38 of aglycone: glucose:acetylglucose: malonylglucose, found in soy flour [7], will be used. As the conjugates vary in weight, it is necessary to calculate the contribution of each form to the total of aglycone equivalents [8], as presented in Eq. (1).

Thus, daidzein conjugates, as weighed out, will contribute 57% aglycone equivalents, with glycitein contributing 60% and genistein 58%. The total isoﬂavone content, obtained after dividing the individual aglycone amounts by the aglycone equivalent 0.57 for daidzein, 0.60 for glycitein and 0.58 for genistein, for the soy seed extract and supplements are presented in Table III.

Discussion

With the accuracy proﬁle method validation, the analytical interpretation is easy and all the useful required statistics, such as trueness, precision and linearity are integrated. In addition, the accuracy proﬁle uses the notion of total error and simpliﬁes the analytical validation procedure while checking the future performances of the procedure [9, 10].

With regard to the seven soy supplements analyzed, important discrepancies were found in the aglycone proﬁles. As shown in Figure 3, each tested sample (S1-S7) revealed a different “fingerprint”.

The soy seed extract and supplements 2 and 3 had a similar proﬁles characterized by small amount of genistein and high levels of daidzein. In supplements 1, 5 and 6 glycitein was not detected, while the highest levels were obtained for daidzein in sample 1 and 5. A similar proﬁle was obtained for supplement 4, where the prevalent aglycone was daidzein, which exceeded the level of glycitein by more than nine times.

This inconsistency is mainly due to the plant part used for obtaining the extract, soy variety, soy crop season, environmental conditions and industrial processing. The soy seed extract contained higher proportions of daidzein and glycitein. This might suggests that the soy extract was obtained from the hypocotyls of the soybean seeds, since these contain the highest levels of daidzein and glycitein.

The low amount found in supplement 5 may be due to the interaction of genistein with the trace minerals present in the supplement’s formulation. Genistein was
found to bind with copper (II) and iron (III) with 1:2 M stoichiometries [11]. The copper and iron chelates are not detectable as they generate a bathochromic shift from 262 nm to 268 nm.

However, important discrepancies between assayed and label content have been reported in other studies assessing soy-based supplements [4,8,12]. At least in part, these variations may be explain by the different ratio of free and conjugated isoflavones found in soy supplements. Since the weight of glucose moiety accounts for approximately 40% of the isoflavone glycoside form, that means that only 60% of the recommended dose represents the biologically active part. So, a soy supplement containing 100 mg isoflavones may contain 60 mg to 100 mg active aglycones depending on the free:conjugated ratio of isoflavones [13].

As presented in Table IV, the isoflavone content of soy supplements is commonly expressed as total isoflavone per dosage form, without any specifications related to the aglycone profile or to the ratio of aglycones to respective glycosides. Supplements 1 and 6 and supplements 4 and 5 claimed to have the same isoflavone content (40 mg and 50 mg, respectively). In each case, different aglycone profiles were obtain, showing that under the same apparent content important differences can be found.

Table III. Isoflavone content in soy samples after acidic hydrolysis and relative differences from the declared isoflavones content.

| Soy supplements (mg dosage form) | Average weight (g) | Individual aglycones D | GY | GN | Aglycone | Isoflavone | Label claim | Bias % |
|---------------------------------|--------------------|------------------------|----|----|----------|------------|-------------|--------|
| S1                              | 0.495              | 7.56                   | 0.00| 4.03 | 11.59    | 20.21      | 20.00       | 1.04   |
| S2                              | 0.333              | 5.78                   | 2.58| 1.37 | 9.73     | 16.81      | 17.50       | -3.95  |
| S3                              | 0.338              | 12.71                  | 6.31| 3.57 | 22.60    | 38.98      | 35.00       | 11.38  |
| S4                              | 1.727              | 13.60                  | 1.56| 8.83 | 23.99    | 41.68      | 50.00       | -16.63 |
| S5                              | 0.551              | 2.45                   | 0.00| 1.03 | 3.48     | 6.07       | 50.00       | -87.86 |
| S6                              | 0.479              | 8.97                   | 0.00| 9.41 | 18.39    | 31.95      | 20.00       | 59.91  |
| S7                              | 0.305              | 4.99                   | 2.10| 2.41 | 9.50     | 16.41      | 12.00       | 36.75  |

Table IV. Statements used on the label for expressing isoflavone content, recommended posology and percentage of NAMS recommended dose for alleviating vasomotor symptoms in postmenopausal women (minimum 50 mg isoflavones daily).

| Sample # | Phrases used to express isoflavone content | Units day\(^{-1}\) | Recommended daily dose (mg day\(^{-1}\)) | % of NAMS recommended daily dose |
|----------|--------------------------------------------|-------------------|------------------------------------------|---------------------------------|
| S1       | “50 mg soy extract (min 40% isoflavones)”  | 2                 | 40                                       | 80                              |
| S2       | “17.5 mg soy isoflavones”                   | 2                 | 35                                       | 70                              |
| S3       | “35 mg soy isoflavones”                     | 2                 | 70                                       | 100                             |
| S4       | “50 mg isoflavones from 125 mg soy concentrate” | 1                | 50                                       | 100                             |
| S5       | “Soy isoflavones 50 mg”                     | 1                 | 50                                       | 100                             |
| S6       | “Soy extract 50 mg (Glycine max) (40% isoflavones)” | 2                | 40                                       | 80                              |
| S7       | “Soy seed extract (Glycine max) 120 mg containing 12 mg isoflavones” | 1-3              | 12-36                                    | 24-72                           |

Dose is assumed to be expressed as total isoflavone content

found to bind with copper (II) and iron (III) with 1:2 M stoichiometries [11]. The copper and iron chelates are not detectable as they generate a bathochromic shift from 262 nm to 268 nm.

However, important discrepancies between assayed and label content have been reported in other studies assessing soy-based supplements [4,8,12]. At least in part, these variations may be explain by the different ratio of free and conjugated isoflavones found in soy supplements. Since the weight of glucose moiety accounts for approximately 40% of the isoflavone glycoside form, that means that only 60% of the recommended dose represents the biologically active part. So, a soy supplement containing 100 mg isoflavones may contain 60 mg to 100 mg active aglycones depending on the free:conjugated ratio of isoflavones [13].

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Given that aglycones are not similar in their estrogenic activity, pharmacokinetics and metabolism, any change in aglycone profile is equivalent to different estrogenic activity and bioavailability.

Although genistein or daidzein estrogenic proprieties were more explored, soy extracts contain mostly glycosides or esterified glycosides. Genistein and diadzein glycosides are not estrogenic and deconjugation is required to release the bioactive aglycones [14]. In vivo, deconjugation of glycosides forms is mainly carried out by the gut microbiota and represents a rate-limiting step in their bioavailability [15]. Therefore, to give an indication of the potential biological effect, the aglycone levels should be labeled rather than the total weight of isoflavones.

Most studies assessing the role of soy isoflavones in alleviating menopause symptoms have focused on genistein, as is the most potent soy isoflavone with regard to receptor binding and transactivation [16,17]. But according to the chromatographic profiles, daidzein was the predominant aglycone in all soy supplements. Its binding affinity towards estrogen receptors has been evaluated to be more than ten times weaker than that of genistein [16]. Moreover, using the immature rat uterotrophic assay, five commercial soy extracts with considerable diversity in isoflavone composition revealed different estrogenic potencies [18]. The study concluded that soy extracts with appreciable amounts of genistein or glycitein presented higher estrogenic potency compared to the ones rich in daidzein.

Despite years of research, the soy efficiency
in alleviating menopause symptoms still represents a controversial subject. The inconsistent results might be due to the little attention paid to determining the exact aglycone profile of the employed soy extracts.

It has been proved that isoflavone supplements containing 15mg or more of genistein and 50mg total isoflavones were efficacious in reducing hot flash symptoms, whereas those providing similar amounts of total isoflavones, but lower amounts of genistein were not [19]. In addition to this, the main results of a large review suggested that soy extracts with high (>30 mg day\(^{-1}\)) levels of genistein consistently reduced the frequency of hot flushes [20]. Another multi-center, randomized, placebo-controlled study concluded that a single dose of 30 mg day\(^{-1}\) of synthetic genistein reduces menopausal symptoms without producing adverse effects [21]. With regard to these, none of the tested supplements provided more than 30 mg of genistein day\(^{-1}\), although they are all recommended for alleviating menopause symptoms.

As genistein represents the most reviewed soy isoflavone, with known pharmacokinetics and metabolism, included in most trials aiming to evaluate soy efficiency in different diseases, standardization of soy supplements in genistein would provide a more accurate indication about the effectiveness of soy supplements in reducing menopause symptoms.

Another concern targets the required daily intake of isoflavones to obtain health benefits during menopause. For alleviating vasomotor symptoms in postmenopausal women, the North American Menopause Society (NAMS) report, published in 2011, recommends a starting isoflavone dose of 50mg or higher per day and that therapy should be given for at least 12 weeks [22]. On this basis, only supplements S3, S4 and S5 recommended an adequate daily dose of isoflavones (Table IV).

In supplements 1 and 6, the presence of other plant extracts might compensate for the lower isoflavone dose. Still, only soy isoflavones can induce estrogenic effects, while the other botanicals exert mainly antioxidant or serotonergic effects. The cumulative effect of these plant extracts remains to be investigated for designing efficacious formulations in reducing menopause symptoms.

None of the tested supplements stated any directions regarding the length of isoflavone supplementation.

**Conclusions**

A comprehensive analysis of isoflavone concentrations in seven soy supplements and one soy seed extract was carried out using a HPLC/UV method for the quantification of daidzein, glycitein and genistein, the main isoflavone aglycones. The proposed method can be easily adapted to routine quality control procedures while the validation strategy takes into account the risk associated with the future use of the method.

Considerable variations in the isoflavone profiles of the analyzed soy supplements were found. Solely reporting the total isoflavone content may be improper, as individual isoflavones have different estrogenic potency and bioavailability.

The problem of expressing isoflavone content has been discussed at the Fifth International Symposium on the Role of Soy in Preventing and Treating Chronic Disease [13]. Although the committee recommended that manufacturers should express the isoflavone content as aglycone amount, the problem of incorrect labeling still persists.

The use of soy supplements standardized in total isoflavones could be an important cause of heterogeneity among clinical trials results. To properly evaluate the efficiency of soy supplements in reducing menopause symptoms, standardization should be made in genistein. Once the issue of soy composition will be straightened out, the results of clinical trials will be more reliable and will either prove or disprove the efficacy of soy supplements in alleviating menopausal symptoms.

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