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Isoflavones in food supplements: chemical profile, label accordance and permeability

study in Caco-2 cells

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Abstract

Consumers nowadays have an active role in their health-care. A special case is the increasing number of reluctant women to the use of exogenous hormone therapy for treatment of menopausal symptoms and looking for complementary therapies. However, food supplements are not clearly regulated in Europe. EFSA only recently begun to address the issue of botanical safety and purity regulation, leading to variability of content, standardization, dosage, and purity of available products.

In this work, isoflavones (puerarin, daidzin, genistin, daidzein, glycine, genistein, formononetin, prunetin, and biochanin A) from food supplements (n=15) for menopausal symptoms were evaluated and compared to the labelled information. Only four supplements comply with the recommendations made by the EC on the tolerable thresholds. Intestinal bioavailability of these compounds was investigated using Caco-2 cell. The apparent permeability coefficients of selected isoflavonoids across the Caco-2 cells were affected by the isoflavone concentration and the product matrix.

Keywords: Food supplements; menopause; isoflavones; HPLC-DAD; bioavailability; Caco-2 cells.
Introduction

Nowadays, consumers are aware of their health and self care. They perform an active role by selecting specific foods and supplements that could improve their health and quality of life. Food supplements (also known as dietary or nutritional supplements) are generally used to overcome nutritional deficiencies, prevent or reduce the risk of disease and/or to promote general well-being. According to European Food Safety Authority (EFSA), food supplements are defined as concentrated sources of nutrients or other substances with a nutritional or physiological effect, whose purpose is to supplement the normal diet. The wide and steadily growing consumption range and popularity of food supplements, constitutes a challenge for consumer protection. Food supplements are not subjected to rigorous standardized and quality control measures, unlike pharmaceuticals, and, therefore, the presence of impurities, adulteration and/or dosage inconsistency can occur. Also, the field of supplements is not clearly regulated in Europe. The number of substances other than vitamins and minerals used in food supplements on the European market is estimated to be over 400, grouped in six main categories: amino-acids, enzymes, prebiotics and probiotics, essential fatty acids, botanicals and botanical extracts and other substances (such as isoflavones)\(^1\). The European Commission has established harmonised rules to ensure that food supplements are safe and properly labelled. However, as these supplements are regulated as foods, the legislation only focuses on vitamins and minerals as ingredients\(^2\). The Directive sets out labelling requirements and requires that EU-wide maximum and minimum levels are set for each vitamin and mineral added to supplements.

The consumer’s lack of information related with composition, \textit{in vivo} absorption and effects are bearing in mind, leading to some doubts. Thus, it is very important to control the market and have a clear idea about their safety.
The consumption of food supplements-containing phytoestrogens among postmenopausal women is rapidly increasing due to their beneficial effects, especially for relief of hot-flushes. Isoflavones are phenolic compounds with antioxidant activity and structural similarity to estradiol molecule, being primarily found in plants of the Fabaceae family, including soy, lentils, bean plant, chickpeas, alfalfa and red clover. A number of epidemiological studies associate the consumption of isoflavone-rich foods with low incidence of the major hormone-dependent cancers, cardiovascular diseases, osteoporosis, and climacteric complaints. Driven by these purported health benefits, a plethora of products containing isoflavones is on the market, specifically targeting women in menopause. These preparations generally contain extracts from soy, red clover and kudzu, as single ingredients or are multi-ingredient formulations mixed with minerals, vitamins, other plant extracts and omega-3, 6, and 9 fatty acids. Soybean (Glycine max (L.) Merril) has in its composition mainly isoflavone aglycones (daidzein, glycitein and genistein) and glycoside, acetylglycoside and malonylglycoside forms. In contrast to soybean, red clover (Trifolium pratense L.) contains biochanin A and formononetin (aglycones), and their glycosides and malonyl derivatives, as the major components. Pueraria mirifica Airy Shaw et Suvatabhandu (Fabaceae), commonly known as White Kwao Krua and Thai kudzu, is an indigenous herb from Thailand, traditionally used in folk medicine for rejuvenation and to attenuate menopausal symptoms. The dried powder of the plant tubers has also been used to prepare food supplements. Several isoflavonoids have been identified in P. mirifica tubers, including glycoside forms (daidzin, puerarin and genistin) and the aglycone forms (daidzein and genistein). Isoflavones, like the majority of polyphenols, are usually found in plants mainly as glycosides and glycoside esters. After ingestion they are metabolised by bacteria in the gastrointestinal tract, releasing their aglycones, the truly bioactive constituents. The Caco-2 monolayer
model has been well recognized for investigation of intestinal transport of xenobiotics \cite{13-14}. The transepithelial transport of flavones occurs by passive diffusion \cite{15}. Thus, it is very important to know the bioavailability of such compounds in order to understand their biological activity in food supplements. Several Caco-2 cell line studies have examined the intestinal absorption of isoflavones as pure compounds \cite{16-17}, but there are very few studies regarding the extracts of isoflavones-containing food supplements \cite{18}. The great variability of products in the market regarding the concentration and source of isoflavones, and its therapeutic uses need more studies \cite{19}. Additionally, the biological effectiveness of these bioactive compounds greatly depends on the intestinal bioavailability, being variable between the different isoflavones.

The aim of this study was to study the isoflavone composition (puerarin, daidzin, genistin, daidzein, glycinein, genistein, formononetin, prunetin, and biochanin A) of 15 commercial food supplements. The obtained results were compared with the labelled information. The permeability of the compounds in some supplements was also assessed using a human colon adenocarcinoma Caco-2 cell line.

2. Material and methods

2.1 Chemicals and reagents

Puerarin (≥99%), daidzin (≥95%), genistin (≥95%), glycinein (≥97%), daidzein (≥98%), genistein (≥98%), biochanin A (≥97%), prunetin (≥98%), and formononetin (≥99%) and the internal standard 2-methoxyflavone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Preparative C\textsubscript{18} sorbent (125 Å, 55-105 µm) was from Waters (Milford, MA, USA). Water was purified with a “Seradest LFM 20” system (Seral, Ransbach-Baumbach, Germany). The eluents were filtered through 0.45 µm filters and degassed under reduced pressure. Disposable acetated cellulose 0.45 µm were from OlimPeak, Teknokroma (Barcelona, Spain). HPLC grade
solvents, methanol, dimethyl sulfoxide (DMSO) and acetonitrile, and analytical grade formic acid were from Merck (Darmstadt, Germany).

Caco-2 (ATCC HTB-37, passage 30-40) human colon adenocarcinoma cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Dulbecco’s Modified Eagle’s Medium (DMEM) with 4.5 g/L glucose and GlutaMAX™, fetal bovine serum (FBS), 0.05% trypsin–EDTA, penicillin–streptomycin (Lonza Biowhitaker, Verviers, Belgium) and non-essential amino acids (NEAA) were obtained from Gibco (Life Technologies, Paisley, UK). Phosphate buffer saline (PBS) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tissue-treated inserts (high density PET membrane, 23.1 mm, 0.3 μm pore size, 4.2 cm²) were from Becton Dickinson Falcon™ (Bedford, MA, USA) and 6 wells plates were purchased from Orange Scientific (Braine-l’Alleud, Belgium).

2.2 Standards

Purity-corrected individual isoflavones stock solutions were prepared in DMSO (1 g/L) and then serially diluted in water: methanol (10:90, v/v) in order to obtain the standard concentrations for the calibration curves. A working 2 mg/L solution of the internal standard was also prepared in DMSO. All solutions were stored in amber glass vials at 4 °C.

2.3 Samples and sample preparation

2.3.1 Sampling

Fifteen different food supplements containing isoflavones were purchased from local retail, herbal stores and pharmacies. Their selection was based on the availability on the market and the range of isoflavone profiles and concentrations. Characteristics of the selected food supplements are presented in Table 1.
The supplements in evaluation were in the form of tablets or capsules and ten from each product were sampled. Tablets were ground to a fine powder using a glass mortar and pestle, after careful removing of the coating, if present. The shells from hard-gelatine capsules were removed and the content homogenised. The content of soft gel capsules were squeezed into a test tube and homogenised.

2.3.2. Matrix solid-phase dispersion

The compounds were extracted based on the procedure described by Visnevschi-Necrasov et al. \( ^{20} \), with several modifications. An aliquot of 0.5 g of the previously homogenized samples, 2 g of C\(_{18}\) and the internal standard (2 mg/L, 0.5 mL) were placed in a glass mortar and blended together using a glass pestle to obtain a complete disruption and dispersion of the sample on the solid support. After complete blending, the sample was packed into an empty column containing a polyethylene frit at the bottom. A second frit was placed on the top of the sample by careful compression with a syringe plunger. The packed column was attached to a vacuum manifold (Visiprep, Supelco) coupled with a vacuum pump and the flow adjusted to 1 mL/min. The column was rinsed with 10 mL water (discharged fraction) and the elution step was carried out with 2 × 5 mL of water: methanol (10:90, v/v) (collected fraction). Before HPLC analysis, extracts were collected in amber vials and filtered through disposable 0.45 μm cellulose membranes. Sample extraction was performed in triplicate.

2.4. HPLC equipment

The chromatographic analysis was performed using an HPLC unit (Jasco, Tokyo, Japan), consisting of two Jasco PU-2080 Plus HPLC pumps, an AS-950 automated injector (20 μL loop), and a MD-2010 Plus multiwavelength diode-array detector (DAD). The separation of the
Isoflavones was carried out on a reversed-phase Luna C18 column (4.60 mm × 150 mm, 5 µm particle size) from Teknokroma (Barcelona, Spain), maintained at 40 °C. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The gradient program was previously developed, and used with some modifications: 0 min 0% B, 15 min 32% B, 18 min 45% B, 23 min 50% B, 25 min 70% B, 35 min 10% B, maintaining these conditions for 5 min and returning to the initial conditions within 3 min. The flow rate of the mobile phase was 1 mL/min and the injection volume was 20 µL.

Peak purity measurements of all compounds were based on spectral comparison at three different peak heights through DAD information. Analytes were monitored at 254 nm and quantified on the basis of the internal standard method. Chromatographic data were processed with ChromNAV Software (Jasco, Tokyo, Japan).

**2.5 Caco-2 cell culture**

Caco-2 cells were obtained from ATCC. Cells were routinely cultured in 75-cm² flasks in Dulbecco’s modified Eagle’s medium (DMEM) containing D-glucose (4.5 g/L) and GlutaMAX™ and supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin (10,000 U/mL), and 1% MEM Amino Acids. The cells were maintained in a humidified atmosphere of 5% CO₂/95% air at 37 °C, and were supplied with fresh medium every 2 days. Cells were subcultured at 80-90% confluence. For experiments, Caco-2 cells were seeded in cell culture inserts in 6-well plates at a density of 4×10⁴ cells/cm². The basolateral and apical compartments contained 2.5 and 1.5 mL of culture medium DMEM, respectively. Culture medium was replaced every day. The integrity of the Caco-2 cell monolayer was checked by Transepithelial Electrical Resistance (TEER) measurements using an epithelial voltammeter (EVOM, World Precision Instrument, Sarasota, FL, USA) for the whole period.
2.6 Permeation experiments

Experiments, in triplicate, were performed 21 days after seeding. The cell monolayers were pre-equilibrated with fresh PBS, pH 7.4 at 37 °C, for 30 min, and the incubation medium was then discarded. Afterwards, 1.5 mL of the test solutions, prepared by dilution of the MSPD extracts in PBS, were added to the apical side of the Caco-2 monolayers and 2.5 mL of PBS to the basolateral side, and allowed to permeate for 120 min at 37 °C under 5% CO₂ and 95% of relative humidity. Samples (0.5 mL) were withdrawn from the receptor side at 0, 15, 30, 60, and 120 minutes for the determination of the isoflavone molecules transported across the monolayer. After each sampling time, the basolateral side was replenished with the same PBS volume. Samples were preserved at -20 °C for subsequent HPLC analysis as described in section 2.4.

The apparent permeation coefficient (P_{app}) of the isoflavones, expressed in cm/s, was calculated from the following equation: P_{app} = Q / (A x C x t), where Q is the total amount of permeated isoflavones during the 120 minutes of experiment (μg), A is the diffusion area (cm²), C is the donor compartment concentration at time zero (μg/mL), and t is the time of experiment (s). In addition to P_{app}, the percentage of permeation (%) of each aglycone (daidzein, genistein, formononetin and biochanin A) was calculated as the proportion of the original amount that permeated through the monolayer, which was calculated as the amount transported divided by the initial amount in the apical chamber.

3. Results and Discussion

The extraction of native isoflavones from natural matrices is commonly based on solvent extraction with polar organic solvents (methanol, ethanol, acetonitrile, acetone, and water, mixed or not), followed (although not always) by a clean-up step to eliminate interfering compounds. Isoflavones are analysed in their conjugated forms or as aglycones, depending
upon the objectives of the research. When a hydrolysis step is employed, the unstable malonyl and acetyl derivatives are converted into more stable forms such as β-glycosides and/or aglycones. At the same time, the chromatographic complexity is reduced. On the other hand, a possible drawback is the increase of the analysis time and/or the possible degradation of compounds. The knowledge of the original isoflavone profile of food supplements is important, since their therapeutic potential seems to depend from the levels and the composition of the isoflavones present.

The use of MSPD for sample preparation is a recent and increasing approach due to the flexibility and versatility of the process, which allows sample extraction and clean-up in one single step. MSPD has been used for the extraction of pesticides, pharmaceuticals, and other contaminants, but also in the isolation of naturally-occurring compounds from different plants including the extraction of isoflavonoids from leguminous plants.

The MSPD methodology used in this study for isoflavones analysis was based on the procedure described by Visnevschi-Necrasov et al. with several modifications. Method optimization was performed in order to reduce the use of organic solvents. As it is well known, sustainability is a recent concept that should be taken into account and encompasses the type of procedures, chemicals and solvents used for the extraction of interesting compounds. For this reason, and bearing in mind the question of green chemistry, the extraction process should be designed as simple as possible and, simultaneously, considering the effective costs for industries. For extraction, the dichloromethane-methanol mixture (25:75, v/v) was replaced by methanol-water (90:10, v/v), a greener mixture of solvents than the previous one, also described as adequate for isoflavonoids extraction. The modified methodology was validated to evaluate its efficiency for isoflavones analysis in food supplements.

The set of standards used was chosen to represent the main isoflavonoids found in soy, red clover and Thai-kudzu products: puerarin, daidzin, genistin, daidzein, glycitein, genistein,
formononetin, prunetin, and biochanin A. The retention times, linear regression data, limit of
detection (LOD) and limit of quantification (LOQ) values of the nine isoflavones investigated
are presented in Table 2.

Standard curves were obtained by plotting standard solutions, at five concentrations (mg/mL
of injected solution), as a function of the ratio between the peak areas of each standard and
the internal standard. Standards were subjected to the entire extraction method and injected
in triplicate. Standard curves were linear over the concentration range tested, with
determination coefficients greater than 0.998 for all the analytes. Relative standard deviations
(RSDs) of the triplicate injections varied between 0.4 and 4.1 %. The deviation in the retention
time was less than 0.06%. The LOD and the LOQ for each isoflavone were calculated as 3.3 and
10 times the standard deviation of the background noise divided by the slope of the calibration
curve, respectively. The obtained LOD values were in the range of 12.6 – 161 ng/g and the LOQ
values ranged from 41.8 to 535 ng/g. For validating analytical accuracy (in terms of recovery),
0.5 g of samples (in duplicate) S10 (soy), RC (red clover), and TK (Thai-kudzu) were spiked with
0.1 mg of all isoflavones, and extracted as previously described. For the three samples, the
mean recovery for all isoflavones ranged from 90.1 to 102.0%, whereas calculated relative
standard deviations (RSD) were below 6.0% (Table 3).

Insert Table 2

Insert Table 3
The results indicate that the MSPD extraction method was accurate and precise. Chromatograms of a standard solution containing the isoflavones puerarin, daidzin, genistin, dadzein, glycitein, genistein, formononetin, prunetin, and biochanin A (A) and of a sample (B) are depicted in Figure 1.

The proposed method was applied to the quantification of 9 isoflavones (aglycones and glycosides) in fifteen food supplements indicated for the management of menopause symptoms, in capsules and tablets, described in Table 1. The determined amounts of the individual isoflavones in the samples (mg/unit), total isoflavones (sum of the individual isoflavones) and the percentage of total isoflavones with respect to the stated content given in the label are presented in Table 4.

The total isoflavones content per unit ranged from 0.029 to 110.9 mg. The percentages of isoflavones with respect to the stated content ranged from 42% to 139%, except for S7 and TK, with 9 and 180% of the label claim, respectively. Label claims, in the majority of the assayed samples, were inconsistent with the determined isoflavones content, with only four food supplements (S9, S10, S12, and RC) compliant with the recommendations made by the European Community on the tolerable thresholds of the claimed constituent content (80–145%) \[27\]. Nevertheless, it is important to state that only 9 isoflavones (aglycone and glycoside forms) were quantified, due to the fact that those are the major compounds in these types of matrices. Other isoflavone derivatives, including malonyl and acetyl derivatives, could also be
present, but were not considered in this study. Recently, Yanaka et al. have also identified isoflavone succinyl glucosides in soy-based products. The isoflavones content described on the labels of all evaluated supplements analysed were unclear (the isoflavone forms included in the total values were not always specified), being difficult to compare labelled values with experimental ones.

The isoflavone profiles of the food supplements revealed a large variability, namely among products containing soy. The concentration of individual isoflavones is labelled in four preparations (Table 4). It is possible to conclude that there are considerable differences between the determined and the claimed content for all the individual isoflavones. The relative amounts of the compounds in the food supplements will depend on the isoflavone composition in primary raw material and the tissue of the soybean from which they are derived. The isoflavone contents of the soy germ and the soy extract used in food supplements are different, with germ containing typically at least about three times the isoflavone content of the whole seed. The soy germ is richer in daidzin and glycitin, while in the cotyledons genistin is the main component. Since differences in the biological activity of the individual isoflavones are recognized, the registered variability in the relative amounts of the different isoflavones may have a considerable impact on the efficacy of the soy-based supplements.

Soybeans and foods derived from soy are major food products for Asian populations, and have been linked to a variety of health outcomes (including low incidence rates of breast and prostate cancers, and reduction of menopause symptoms). Several works have estimated that the dietary intake of isoflavones by those populations range between 30 and 50 mg/day (expressed as aglycone equivalents). Therefore, such studies provided the background for many clinical researches about the effects of isoflavones consumption, which have used supplements containing at least 40 mg of isoflavones. According to the recommended daily doses described in the labels only five samples (S3, S9, S12, RC, and SR) can provide the daily
amount of isoflavones, with samples S9 and S12 supplying two times or more that level. Previous works on the quantification of isoflavones in food supplements have also reported inconsistencies in the isoflavone content from that claimed by the manufacturers. Setchell et al. analysed 33 supplements containing isoflavones and revealed significant differences between labelled and determined isoflavone contents, with approximately half of the supplements presenting lower isoflavones content than the indicated one. Nurmi et al. analysed fifteen soybean-based supplements available in Finland and found only one that had the content mentioned on the label, with the remaining products presenting isoflavones content lower than claimed. Recently, Clarke et al. studied 35 food supplements available in the UK, Canada and Italy and concluded that the preparations evaluated did not contained the claimed content and only 14 food supplements were found to deliver more than 40 mg/day of aglycone isoflavones. Similar outcomes were reported by Boniglia et al. after analysing 14 food supplements intended for menopausal symptoms, available in Italy. In this study, soy aglycones were determined after hydrolysis, and it was concluded that in more than 50% of the analysed products, isoflavones contents were below those claimed. This study confirms the need for more rigorous control on the labelling of food supplements.

The most studied species for the treatment of menopause symptoms and used in supplements are soy and red clover. When consumed, isoflavones are hydrolyzed not only by gastric acid but also by bacterial glucosidases of human intestinal microflora. Sugar moieties are cleaved and aglycones (the bioactive form) are released, which can be absorbed intact by enterocytes or further biotransformed by bacteria to specific metabolites.

In order to evaluate the in vitro bioavailability and distribution of the main isoflavone aglycones, a permeation study was performed using the well-established Caco-2 cell monolayers for three food supplements: S1 (standardized isoflavonoids from soy), S6 (soy extract), and RC (red clover). These supplements were selected because soy and red clover are
generally the main source of isoflavones used in those products. Also, we search for differences among soy supplements with different treatments (pure soy isoflavones and a soy extract). The Caco-2 cell model provides a robust manner to measure the ability of compounds to be absorbed from small intestine and, in addition, to compare the absorption between the different supplements. Test solutions were prepared to contain 1 mg/mL of the respective product. The permeation of the selected isoflavones presented in the food supplements, through the Caco-2 cell model, is depicted in Figure 2 as cumulative transport over time along 120 minutes, simulating digestion time. It is evident that isoflavones permeation through Caco-2 cell monolayer increased over time for all isoflavones. After 120 minutes, the basolateral recoveries were for daidzein: 5.80 ± 0.48% (S1); 7.15 ± 0.43% (S6); 5.47 ± 0.53% (RC); genistein: 2.70 ± 0.37% (S1); 4.75 ± 0.43% (S6), 8.93 ± 0.46% (RC); formononetin: 27.8 ± 1.52%(RC) and for biochanin A 27.9 ± 2.32 % (RC).

Table 5 summarizes the isoflavones apparent Papp in the Caco-2 model. In the red clover supplement (RC), the permeability of formononetin and biochanin A were similar. For the three analysed supplements the Papp for genistein was considerably lower for supplement S1. This may be due to the complexity of the extract, as the extracted matrix also possesses additional components responsible for the diffusion saturation, resulting in the delay of permeability kinetics for genistein. Moreover, the commercial products also contain mucilage on their composition, which may act as a physical barrier against the free diffusion of compounds through the epithelia layer, justifying the Papp differences between different extracts. As Wang et al., who studied red clover food supplements, the present results concluded that the permeability of formononetin was similar to that of biochanin A.
4. Conclusions

Food supplements acceptance by consumers continues to grow in Europe, but there is a lack of regulation that needs to be improved. The isoflavones quantification in food supplements is important for the quality control of these products, since they are often used by consumers for medicinal purposes. In this way, it is very important to ensure that labels are in accordance with the real composition of the product. In this study, 15 commercial food supplements were analysed regarding its isoflavone qualitative and quantitative composition. The proposed method to quantify isoflavones in food supplements was based on a MSPD extraction in the presence of an internal standard, followed by the direct analysis by HPLC/DAD. This method was used taken into account sustainability questions regarding the chemicals and solvents employed. The obtained results showed significant differences between labelled and determined contents for the majority of food supplements.

One of the most important factors to define the oral absorption should be the permeability across the intestinal membrane. Therefore, it is crucial to evaluate in vitro cell models in order to obtain a better correlation with in vivo data, which will be set in our ongoing research work. In this study, we reported the permeation of isoflavones from different food supplements in Caco-2 cells. The apparent permeability coefficients (Papp) of the isoflavonoids across the Caco-2 cell monolayers were found to be affected by the isoflavone concentration and the product matrix.

Reliable labelling information, better standardization, improved manufacturing practices and regulation of the market is required to assure the isoflavone supplements quality. This study reinforces the need for careful selection of isoflavone-containing food supplements by consumers, retailers and health care professionals.
Conflict of interest

The authors declare no conflict of interest. This article does not contain any studies with human or animal subjects.

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References

Figure Captions

Figure 1 - Chromatograms (254 nm) of a standard mixture (A) and of sample S1 (standardized isoflavonoids from soy) (B).

Figure 2 - Transport of the isoflavones daidzein, genistein, formononetin, and biochanin A in three food supplements across Caco-2 cell monolayer: (♦) S1 (isoflavonoids from soy dosed at 30 mg/g), □ S6 (soy extract), ▲ S11 (red clover extract). Data (means ± SD; n=3) are expressed as percentage of the amount of the isoflavones applied to the apical side of the cell culture inserts.
Figure 2

Daidzein

Formononetin

Genistein

Biochanin A
Tables Caption

Table 1 - Sample code and specifications of the analysed food supplements as provided by the manufacturers.

Table 2 - Retention times (t_R), linear regression data, LOD and LOQ values of the nine isoflavones investigated.

Table 3 - Recoveries of the nine isoflavones from soy, red clover, and Thai-kudzu samples. Values are mean ± SD, n=3.

Table 4 - Isoflavones content in the 15 food supplements analysed (mg/unit) and percentages of total isoflavone with respect to the labelled content.

Table 5 - Apparent permeability coefficient (Papp) (apical to basolateral) for food supplements extracts across the Caco-2 monolayer.
<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Origin and composition</th>
<th>Dosage form</th>
<th>Capsule/tablet weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>EU. Natural isoflavonoids from non-transgenic soy.</td>
<td>Capsule</td>
<td>0.394</td>
</tr>
<tr>
<td>S2</td>
<td>France. Soy extract (<em>Glycine max</em>), excipients.</td>
<td>Capsule</td>
<td>0.334</td>
</tr>
<tr>
<td>S3</td>
<td>France. Tomato extract, milk proteins, soy extract, vitamin C.</td>
<td>Capsule</td>
<td>0.740</td>
</tr>
<tr>
<td>S4</td>
<td>United Kingdom. Evening primrose oil, soy isoflavones, fish oil, vitamin E, excipients.</td>
<td>Gel capsule</td>
<td>0.564</td>
</tr>
<tr>
<td>S5</td>
<td>UE. Coral Calcium, soy extract rich in phytoestrogens, Passiflora, excipients.</td>
<td>Tablet</td>
<td>1.237</td>
</tr>
<tr>
<td>S6</td>
<td>France. Yam extract (<em>Dioscorea opposita</em> Thunb), soy extract (<em>Glycine max</em>), fructo oligo saccharides, hop (<em>Humulus lupulus</em>), meadowsweet (<em>Spiraea ulmaria</em>), grape vine (<em>Vitis vinifera</em>), vitex (<em>Vitex agnus castus</em>), vitamin E, selenized yeast.</td>
<td>Capsule</td>
<td>0.307</td>
</tr>
<tr>
<td>S7</td>
<td>Portugal. <em>Dioscorea opposita</em>, wild yam, Soy (<em>Glycine max</em>) (pure isoflavones), primrose oil, Dong Quai (<em>Angelica sinensis</em>), melissa (<em>Melissa officinalis</em>), sage (<em>Salvia officinalis</em>), siberian ginseng (<em>Eleutherococcus senticosus</em>), hop (<em>Humulus lupulus</em>), vitex (<em>Vitex agnus-castus</em>), vitamins E, B6.</td>
<td>Gel capsule</td>
<td>0.470</td>
</tr>
<tr>
<td>S8</td>
<td>Spain. Red algae (<em>Lithothamnium calcareum</em>), fermented soy, soy isoflavones.</td>
<td>Capsule</td>
<td>0.565</td>
</tr>
<tr>
<td>S9</td>
<td>EU. Evening primrose oil (<em>Oenothera biennis</em>), soy isoflavones, vitamin E, excipients.</td>
<td>Gel capsule</td>
<td>0.447</td>
</tr>
<tr>
<td>S10</td>
<td>Belgium. Sage extract, soy extract, saffron (<em>Crocus sativus</em>), vitamin B6.</td>
<td>Tablet</td>
<td>0.772</td>
</tr>
<tr>
<td>S11</td>
<td>EU. Soy isoflavones, sage, oat, marine magnesium, vitamin E, excipients.</td>
<td>Capsule</td>
<td>0.538</td>
</tr>
<tr>
<td>S12</td>
<td>Italy. Soy isoflavones (with 55-72% genistin/genistein), excipients.</td>
<td>Capsule</td>
<td>0.623</td>
</tr>
<tr>
<td>RC</td>
<td>Australia. Standardized red clover (<em>Trifolium pratense</em>) extract, excipients.</td>
<td>Tablet</td>
<td>0.351</td>
</tr>
<tr>
<td>SR</td>
<td>Spain. Soy rich in isoflavones of retarded action, Yam extract, red clover extract, vitamins A, C, B1, B2, B12, E, excipients.</td>
<td>Capsule</td>
<td>0.634</td>
</tr>
<tr>
<td>TK</td>
<td>USA. Vitamin B12, standardized <em>Pueraria mirifica</em> root extract (Thai Kudzu): miroestrol, isoflavonoids; pyridoxal-5 phosphate, biotin, folic acid, excipients.</td>
<td>Capsule</td>
<td>0.519</td>
</tr>
</tbody>
</table>
Table 2

<table>
<thead>
<tr>
<th>Isoflavones</th>
<th>tᵣ (min)</th>
<th>Regression equation</th>
<th>Linear range (mg/mL)</th>
<th>R²</th>
<th>LOD (ng/g)</th>
<th>LOQ (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puerarin</td>
<td>11.287</td>
<td>y = 29.933x + 0.0217</td>
<td>0.010-0.050</td>
<td>0.9995</td>
<td>23.9</td>
<td>79.8</td>
</tr>
<tr>
<td>Daidzin</td>
<td>12.641</td>
<td>y = 23.197x + 0.1485</td>
<td>0.050-0.250</td>
<td>0.9994</td>
<td>138</td>
<td>459</td>
</tr>
<tr>
<td>Genistin</td>
<td>14.661</td>
<td>y = 24.982x + 0.1742</td>
<td>0.050-0.250</td>
<td>0.9991</td>
<td>161</td>
<td>535</td>
</tr>
<tr>
<td>Daidzein</td>
<td>18.303</td>
<td>y = 35.457x + 0.0103</td>
<td>0.005-0.025</td>
<td>0.9995</td>
<td>12.6</td>
<td>41.8</td>
</tr>
<tr>
<td>Glycitein</td>
<td>18.712</td>
<td>y = 21.774x + 0.0054</td>
<td>0.005-0.025</td>
<td>0.9994</td>
<td>12.9</td>
<td>43.2</td>
</tr>
<tr>
<td>Genistein</td>
<td>20.657</td>
<td>y = 35.871x + 0.0132</td>
<td>0.005-0.025</td>
<td>0.9992</td>
<td>17.9</td>
<td>59.7</td>
</tr>
<tr>
<td>Formononetin</td>
<td>22.367</td>
<td>y = 33.257x + 0.0108</td>
<td>0.005-0.025</td>
<td>0.9994</td>
<td>13.9</td>
<td>46.5</td>
</tr>
<tr>
<td>Prunetin</td>
<td>25.624</td>
<td>y = 39.776x + 0.0119</td>
<td>0.005-0.025</td>
<td>0.9983</td>
<td>22.6</td>
<td>75.4</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>25.799</td>
<td>y = 35.825x + 0.0043</td>
<td>0.005-0.025</td>
<td>0.9982</td>
<td>23.5</td>
<td>78.4</td>
</tr>
</tbody>
</table>

* a, standard peak area/internal standard peak area; x, concentration (mg/mL of injected solution).
<table>
<thead>
<tr>
<th>Compound</th>
<th>Soy Recovery %</th>
<th>Soy RSD %</th>
<th>Red clover Recovery %</th>
<th>Red clover RSD %</th>
<th>Kudzu Recovery %</th>
<th>Kudzu RSD %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puerarin</td>
<td>98.1</td>
<td>2.5</td>
<td>94.3</td>
<td>2.8</td>
<td>96.3</td>
<td>1.5</td>
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<tr>
<td>Daidzin</td>
<td>91.1</td>
<td>0.2</td>
<td>90.1</td>
<td>0.9</td>
<td>94.2</td>
<td>0.7</td>
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<tr>
<td>Genistin</td>
<td>92.4</td>
<td>1.7</td>
<td>95.5</td>
<td>1.3</td>
<td>93.6</td>
<td>0.9</td>
</tr>
<tr>
<td>Daidzein</td>
<td>92.5</td>
<td>3.2</td>
<td>94.2</td>
<td>2.4</td>
<td>102.0</td>
<td>4.8</td>
</tr>
<tr>
<td>Glycitein</td>
<td>94.2</td>
<td>1.1</td>
<td>92.6</td>
<td>2.0</td>
<td>93.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Genistein</td>
<td>101.2</td>
<td>4.2</td>
<td>97.3</td>
<td>3.5</td>
<td>100.8</td>
<td>5.2</td>
</tr>
<tr>
<td>Formononetin</td>
<td>95.0</td>
<td>3.5</td>
<td>96.0</td>
<td>1.7</td>
<td>99.8</td>
<td>0.2</td>
</tr>
<tr>
<td>Prunetin</td>
<td>99.2</td>
<td>2.8</td>
<td>100.5</td>
<td>4.2</td>
<td>100.5</td>
<td>5.2</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>91.5</td>
<td>4.5</td>
<td>95.7</td>
<td>2.9</td>
<td>93.6</td>
<td>6.0</td>
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</table>
Table 4

<table>
<thead>
<tr>
<th>Product type</th>
<th>Sample code</th>
<th>Individual isoflavones, mg/unit</th>
<th>Total isoflavones (mg/unit)</th>
<th>Total labelled isoflavones (mg/unit)</th>
<th>% Labelled isoflavones</th>
<th>RDD</th>
<th>Actual RDD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy</td>
<td>S1</td>
<td>Puerarin 0.90, Daidzin 1.22, Genistin 2.24 (7.75), Daidzein 0.05 (5.20), Glycitein 5.28 (2.02)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>9.7</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>S2</td>
<td>nd 5.80, 1.30, 0.15 (5.5), 0.07, 0.01</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>7.3</td>
<td>17.5</td>
</tr>
<tr>
<td></td>
<td>S3</td>
<td>nd 8.50, 10.16, 0.48, 0.13, 0.08</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>19.4</td>
<td>25</td>
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<tr>
<td></td>
<td>S4</td>
<td>nd 0.47, 0.10, 7.66, 0.13, 16.05</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>24.4</td>
<td>40</td>
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<tr>
<td></td>
<td>S5</td>
<td>nd 23.79, 5.77, 1.68, 0.65, 1.40</td>
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<td>nd</td>
<td>nd</td>
<td>33.3</td>
<td>60</td>
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<tr>
<td></td>
<td>S6</td>
<td>nd 9.86, 2.10, 1.21, 0.64, 0.29</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>14.1</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>S7</td>
<td>nd 3.38, 0.84, 0.13, 0.08, 0.04</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>4.5</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>S8</td>
<td>nd 2.69, 3.82, 12.97, 0.17, 5.83</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>25.5</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>S9</td>
<td>nd 3.83, 5.10, 21.70 (12.8), 0.33 (1.4), 9.08 (20.8)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>40.0</td>
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<tr>
<td></td>
<td>S10</td>
<td>nd 8.69, 18.71, 2.10, 0.48, 2.05</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>32.0</td>
<td>34</td>
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<tr>
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<td>S11</td>
<td>nd 5.97, 2.57, 0.76, 0.29, 0.54</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>10.1</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>S12*</td>
<td>nd 34.00, 69.01, 5.37, 0.42, 2.07</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>110.9</td>
<td>80</td>
</tr>
<tr>
<td>Red clover</td>
<td>RC</td>
<td>nd 0.048, 0.15, 0.58, 0.83, 16.19</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>44.6</td>
<td>40</td>
</tr>
<tr>
<td>Soy + red clover</td>
<td>SR</td>
<td>nd 14.28, 17.02, 13.33, 1.02, 7.33</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>53.3</td>
<td>NA</td>
</tr>
<tr>
<td>Thai-kudzu</td>
<td>TK</td>
<td>0.007, 0.009, 0.009, 0.009</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>0.029</td>
<td>0.016</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation, n=3. Values on brackets correspond to the concentration of individual isoflavones given in label. *, S12 label reports 44-57.6 mg genistein/genistin per capsule; †, the value for total isoflavones refers to the sum of puerarin, daidzin, genistin, daidzein, genistein, glycitein, formononetin, prunetin and biochanin A; ‡, percentage of labelled isoflavones calculated as: total isoflavones/unit /label claim*100; nd, not detected. NA, not available.
Table 5

<table>
<thead>
<tr>
<th>Isoflavones</th>
<th>S1</th>
<th>S6</th>
<th>RC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puerarin</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Daidzein</td>
<td>6.83±0.50</td>
<td>8.86±0.37</td>
<td>6.78±0.66</td>
</tr>
<tr>
<td>Genistein</td>
<td>2.81±0.48</td>
<td>10.70±0.36</td>
<td>11.07±0.06</td>
</tr>
<tr>
<td>Formononetin</td>
<td>nd</td>
<td>nd</td>
<td>1.76±0.10</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>nd</td>
<td>nd</td>
<td>1.07±0.09</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation, n=3; nd, not detected.