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Isoflavones: Vegetable Sources, Biological Activity, and Analytical Methods for Their Assessment

Daniela-Saveta Popa and Marius Emil Rusu

Additional information is available at the end of the chapter

Abstract

Phytoestrogens are natural compounds found in various plant species and they have the ability to bind to the estrogenic receptors, exerting agonist and/or antagonist effects. The main classes of phytoestrogens are isoflavones, lignans, and coumestranes. Isoflavones are plant bioactive nonsteroidal polyphenolic metabolites with antioxidant properties. They have a very close structure with 17β-estradiol and possess estrogenic/antiestrogenic effects. The main dietary source of isoflavones is soy (*Glycine max* L.). Other legumes, such as red clover (*Trifolium pratense* L.), alfalfa (*Medicago sativa* L.), and *Genista* species, have important content in isoflavones, showing nutritional or phytotherapeutic interest. In plants, isoflavones can be found mainly as non-active glycosides which are converted after ingestion, in the corresponding aglycones (e.g., genistein, daidzein) that have pharmacological activity. Many studies have demonstrated the benefits of dietary isoflavones in menopause and multiple chronic pathologies, including cardiovascular diseases, osteoporosis, and hormonal cancers. Dietary intake of isoflavones is widespread, mainly due to the consumption of soybean products. Analytical methods applied for the quantification of isoflavones allow both assessment of dietary intake of isoflavones and highlighting natural sources with phytotherapeutic potential. Health benefits of isoflavones justify the interest for this class of functional food; therefore, further clinical and epidemiological studies are required.

Keywords: nutraceuticals, phytoestrogens, isoflavones, vegetables, analysis

1. Introduction

Phytoestrogens are natural nonsteroidal compounds able to bind to estrogenic receptors and have both estrogenic and antiestrogenic activities. They are widespread in the plant kingdom being considered ubiquitous. The main classes of phytoestrogens are isoflavones, coumestans, and lignans.

Isoflavones are plant-derived secondary metabolites with a polyphenolic structure and antioxidant properties [1]. They pertain to the flavonoid class and are found mostly in plants belonging to Fabaceae family. Soy (*Glycine max* L.) is the major natural source of isoflavones, and the benefits associated with a soy diet occur mostly because of these phytochemicals. Other natural sources of isoflavones are red clover (*Trifolium pratense* L.), alfalfa (*Medicago sativa* L.), and species of the genus *Genista*. All of these plants present phytotherapeutic and nutraceutical significance, and their by-products, herbal teas, and food supplements are often used.

Several epidemiological studies have demonstrated the benefits of dietary isoflavones in menopause and multiple chronic pathologies, including cardiovascular diseases, osteoporosis, and hormonal cancers. The main mechanisms of action of isoflavones, their benefits to human health, and the factors involved in the modulation of their bioactivity are shown in this chapter. Moreover, the analytical methods used for their quantification in plant and food samples are introduced. These are very important methods to evaluate the human exposure to isoflavones and also to assess the optimum intake for human well-being.

2. Characteristics of isoflavones

2.1. Chemistry and metabolism of isoflavones

Isoflavones (IFs) are yellow pigments derived from 3-phenyl-benzopyrone (3-phenyl-chromone) structure. They are found in plants mostly as biologically inactive glycosides: 7-*O*-β-D-glycosides, 6″-*O*-acetyl-7-*O*-β-D-glucosides, and 6″-*O*-malonyl-7-*O*-β-D-glycosides [1, 2]. After ingestion, glycosides are not bioavailable to be absorbed through enterocytes [3]. They are hydrolyzed into bioactive aglycones by both intestinal mucosa and bacterial β-glucosidases from the gut microbiota. Only these forms are absorbed into systemic circulation directly or after subsequent metabolism in the bowel by intestinal bacteria [3]. Soybeans incorporate predominantly genistin, daidzin, and glycitin as inactive glycosides, which are hydrolyzed into their corresponding biologically active aglycones: genistein, daidzein, and glycitein. Other isoflavones observed in legumes are ononin and sissotrin, with their aglycones, formononetin, and biochanin A, respectively (**Figure 1**).

The absorption of aglycones is fast and efficient. Plasmatic isoflavone levels increase up to micromolar-level values after the consumption of soy-based foods, compared to the nanomolar (≤40 nm) levels found in diets without soy [4]. First pharmacokinetic study on isolated and purified isoflavones was performed, when a single dose of 50 mg of aglycone or the equivalent dose of β-glycoside, respectively, was given to healthy adult volunteers. The plasmatic peak values (Cmax) were 341 ± 74 ng/mL for genistein and 194 ± 30.6 ng/mL for daidzein. The times when the values reached the peaks were 5.2 and 6.6 hours (tmax) in the case of direct aglycones ingestion and 9.3 and 9.0 h in the case of the ingestion of β-glycosides, genistin, and daidzin, due to the time required for their hydrolysation. The bioavailability of genistein and daidzein (based on the area under the curve in plasma concentration *versus* time graph) was higher after consumption of β-glycosides [5].

Formononetin and biochanin A can be transformed to daidzein and genistein, respectively, through 4′-*O*-demethylation by the gut microflora or in the liver [6]. Aglycones can be further metabolized through several steps: reduction, deoxygenation, hydroxylation, and C-ring

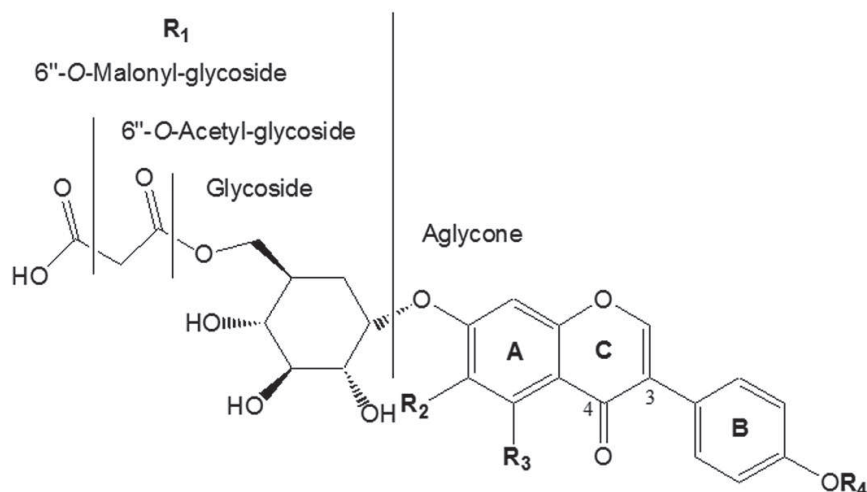| Aglicones	R_1	R_2	R_3	R_4
Genistein	H	H	OH	H
Daidzein	H	H	H	H
Formononetin	H	H	H	CH_3
Glycitein	H	OCH_3	H	H
Biochanin A	H	H	OH	CH_3

Figure 1. Chemical structure of main isoflavones.

cleavage. Daidzein forms S-(−)equol and O-desmethylangolensin (O-DMA) via dihydro-daidzein (**Figure 2**). Similarly, genistein is metabolized first as dihydrogenistein and then as 5′-hydroxy-equol and p-ethyl phenol (**Figure 2**). Another possible minor pathway is the hydroxylation of isoflavone rings at different positions, catalyzed by hepatic cytochrome P450 isoenzymes [2]. Metabolites with phenolic or polyphenolic structures are conjugated to O-glucuronides and sulfate esters during and after absorption through the gut barrier and more intense in the liver. The conjugated metabolites are urinary or biliary excreted and have enterohepatic circulation [4, 7].

Gut microbiota play a very important role in the isoflavone metabolism. The positive effects of a soy-rich diet derive from the existence of microorganisms in the gut capable of intense metabolization of isoflavones. It is the so-called equol producer phenotype, responsible for metabolizing daidzein to equol and identified through the equol/daidzein ratio in the 24-hour urine. Asian people (Japanese, Korean, or Chinese) and Western adult vegetarians are 50–60% equol producers, but equol producers are only 25–30% in Western population. This phenotype is rather stable and cannot be modulated through prebiotic or probiotic nutritional interventions [8]. Otherwise, there are differences between human and animal metabolism, and therefore in vivo results are not relevant to humans [9]. All tested animals had equol in urine after the ingestion of soy or clover [8]. Notably in rodents, equol constitutes 70–90% from the serum isoflavones, compared to humans where only 30% of the daidzein absorbed is metabolized as equol [4].

2.2. Isoflavone content in different sources

Isoflavones can be found in legumes [10–12], nuts, and some fruits, such as currants and raisins [13], coffee [14], and cereals [15], but the most important dietary sources are soybeans and
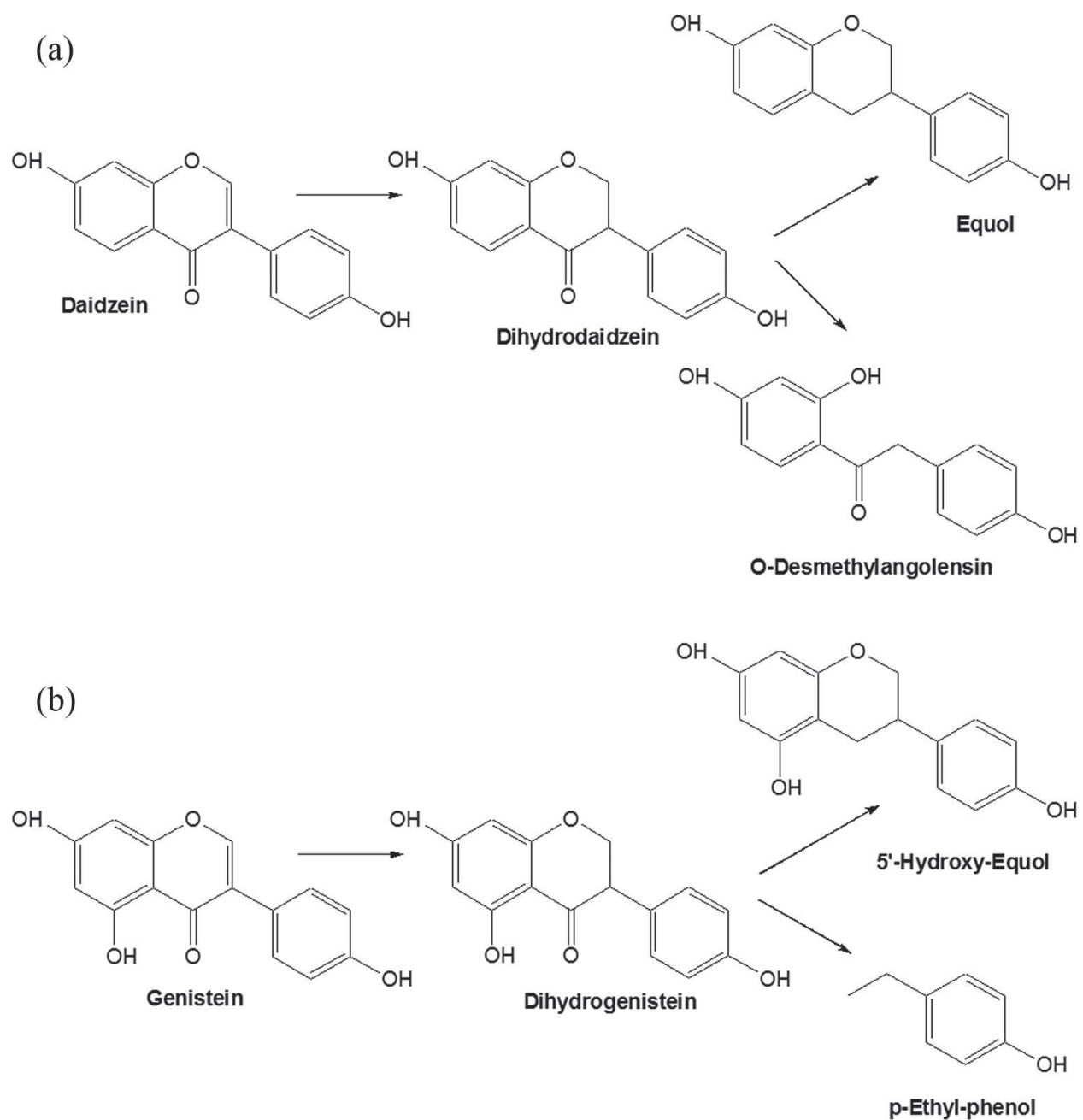
Figure 2. Metabolic pathways of daidzein and genistein.

their by-products [10, 12]. The content of isoflavones in several plants and foods is presented in **Tables 1** and **2**. Soy can be ingested as textured soy protein, as soy milk or drink, added to many fortified foods (e.g., energized bars, cereals, baby formula), or consumed as fermented soybean products, such as miso, natto, and tempeh (**Table 3**) [12]. Also, many food supplements containing soy isoflavones are on the market [16].

Isoflavone content in plants can vary greatly (up to threefold) for the same variety by growth conditions, geographical areas, years, biotic stress factors (e.g., pests), and abiotic stress factors, such as temperature, nutritional status, or drought [4]. Dietary culture has an especially

Food description	Daidzein	Genistein	Glycitein	Total IFs
Soybeans, green, mature seeds, raw	61.70	60.07	7.07	128.83
Soybeans, mature seeds, raw (the mean values from Australia, Brazil, China, Europe, Japan, Korea, Taiwan, the USA)	62.07 (27.77–78.86)	80.99 (39.78–89.32)	14.99 (9.01–22.37)	154.53 (85.68–178.81)
Soybeans, mature seeds, cooked, boiled	30.76	31.26	3.75	65.11
Beans, common, raw (*Phaseolus vulgaris*)	0.29	0.30	0.00	0.59
Beans, adzuki, mature seeds, raw	0.36	0.23	0.00	0.59
Beans, pinto, mature seeds, raw	0.01	0.17	-	0.18
Black bean, sauce	5.96	4.04	0.53	10.26
Chickpeas, mature seeds, raw	0.21	0.06	0.18	0.38
Chickpeas, mature seeds, cooked, boiled	0.00	0.02	-	0.02
Peas, green, split, mature seeds, raw	0.32	0.11	0.00	0.44

Table 1. Isoflavone content in selected legumes (mg/100 g, edible portion—the mean value derived from multiple experiments) [12].

Food description	Coumestrol	Formononetin	Biochanin A
Egg, whole, raw, fresh	0.00	0.05	0.05
Alfalfa seeds, sprouted, raw	1.60	1.43	0.04
Clover sprouts, raw	14.08	3.15	0.59
Red clover	1322.00	833.00	-
Soybeans, mature seeds, raw	0.02	8.46	0.00
Soybeans, mature seeds, sprouted, raw	0.34	0.03	0.00
Lima beans, large, mature seeds, raw	0.14	0.32	0.27
Lima beans, large, mature seeds, boiled	0.00	0.01	0.00
Chickpeas, mature seeds, raw	0.01	0.12	1.54
Chickpeas, mature seeds, canned	0.00	0.00	-

Table 2. Coumestrol, Formononetin, and Biochanin A in selected foods (mg/100 g, edible portion—the mean value derived from multiple experiments) [12].

Food description	Daidzein	Genistein	Glycitein	Total IFs
Miso	16.43	23.24	3.00	41.45
Natto	33.22	37.66	10.55	82.29
Tempeh	22.66	36.15	3.82	60.61
Tofu, raw, regular, prepared with calcium sulfate	8.56	12.99	1.98	22.73
Soybeans, green, raw (includes edamame)	20.34	22.57	7.57	48.95
Soybeans, green, cooked, boiled, drained, without salt (includes edamame)	7.41	7.06	4.60	17.92
Soybeans, mature seeds, sprouted, raw	12.86	18.77	2.88	34.39
Instant beverage, soy, powder, not reconstituted	40.07	62.18	10.90	109.51
Soy cheese, unspecified	5.79	11.14	-	25.72
Soy drink	2.75	5.10	-	7.85
Soy flour (textured)	67.69	89.42	20.02	172.55
Soy meal, defatted, raw	80.77	114.71	16.12	209.58
Soy protein drink	27.98	42.91	10.76	81.65
Soy protein isolate	30.81	57.28	8.54	91.05
Soy yogurt	13.77	16.59	2.80	33.17

Table 3. Isoflavone content in soy foods (mg/100 g, edible portion—the mean value derived from multiple experiments) [12].

big influence on isoflavone content in the diet. Asian and vegetarian diets provide 20–50 mg isoflavones/day, in some cases reaching 100 mg/day, while the Western diet contributes only 0.2–1.5 mg isoflavones/day [2]. Based on recent report of European Food Safety Authority (EFSA), in Europe the dietary isoflavone intake is usually under 1 mg/day, despite an increase in the soy food consumption [17]. The differences between the types of diets refer to the amount of isoflavone in foods, as well as the type of food consumed. In the Western diet, solid processed soy products (such as tofu) and soymilk dominate the diet, and they contain both glycosides (genistin and daidzin which are stable during processing) and aglycones. In the Asian diet, most soy products are obtained through fermentation and have higher amounts of aglycones [3]. Miso, fermented soybean paste (Japan); doenjang, fermented soybean paste (Korea); douchi, fermented soybeans (China); and tempeh, fermented soybean cake (Indonesia) are staple foods in some Asian countries. Simultaneously, health benefit probiotics are formed in these foods during the fermentation processes [18].

Besides soy, other plants in the Fabaceae family have a high content of isoflavones: species of clover, mainly red clover (*Trifolium pratense* L.), alfalfa (*Medicago sativa* L.), and hop clover (*Medicago lupulina* L.), form important part of animal feed. These plants are used in phytotherapy, as medicinal teas or as food supplements. Red clover (*Trifolium pratense* L.) incorporates mainly genistein, daidzein and formononetin, and their respective β-glycosides [19–22]. It also contains important quantities of coumestrol, a phytoestrogen part of coumestan class [19, 20], and antioxidant compounds [23, 24]. Data from scientific literature show that red clover extracts can be used as replacement for conventional hormonal therapy in menopause or hormone-dependent diseases [25]. Alfalfa (*Medicago sativa* L.) contains isoflavones (genistein, daidzein, formononetin, biochanin A) in addition to other phytoestrogens (coumestrol) and many nutrients. It is used in phytotherapy for its antianemic, antihemorrhagic, and remineralization properties [26] and for its hypocholesterolemic, antimicrobial, hypolipidemic, antioxidant, antiulcer, neuroprotective, and estrogenic properties [27]. Species of *Genista* (*G. tinctoria* L., *G. sagittalis* L.) contain essentially genistin and genistein [19, 20, 28, 29]. They are known for their hypoglycemic [30], anti-inflammatory, antiulcer, spasmolytic, antioxidant, and estrogenic properties [31]. Among these plants, *Genista tinctoria* L. show antioxidant and antitoxic activities [32, 33], protective effect against ultraviolet (UV) radiation, and in vitro melanoma cell proliferation [31].

2.3. Mechanism of estrogen-like action of isoflavones

According to the xenohormesis theory, plants synthesize phytochemicals to withstand and adapt under stress. Indeed, isoflavone biosynthesis depends on the environmental conditions in which the plant grows and is stimulated by stress. The stress-induced plant compounds have the ability to upregulate stress adaptive pathways in animals and humans. In the body, the biological effects of isoflavones are exercised by modulating pathways mediated by estrogen receptors (ERs) or various key enzymes involved in cellular signaling or metabolism and antioxidant potential [4].

3. The estrogenic/antiestrogenic effects

Isoflavones produce both estrogenic and antiestrogenic effects through several ways. Due to their structure similar to that of 17β-estradiol, they have the ability to bind to the nuclear ERs, but their affinity for these receptors is rather weak. Only genistein shows stronger affinity for ERβ to which it binds preferentially. Its relative affinity (0.87) is closer to that of the reference hormone, 17β-estradiol. Daidzein affinity for these receptors is 0.005, but equol, its metabolite, has a 5.7 times stronger affinity, thus increasing its estrogenic potential. The affinity for ERα decreases as follows: genistein > equol > daidzein, with the values of 0.04, 0.005, and 0.001, respectively. The affinities of other isoflavones are less than 0.0001 [2, 4].

Isoflavones induce agonist/antagonist effects depending on the level of the endogenous estrogen. For people with high levels of estrogen, (women premenopause, especially in the follicular phase of the menstrual cycle), the isoflavones bind to the estrogen receptors. Because of their weak estrogen potency, isoflavones exert an antagonist effect. They block the action of

endogenous estrogens on their receptors. In case of low concentration of endogenous estrogens (women in menopause, after ovariectomy, or males), the estrogenic action of isoflavones becomes evident, showing additive agonist effect [34]. This is the reason why isoflavones can be used as a long-term complementary or alternative hormone therapy [35].

Isoflavones and their active metabolites can bind to the membrane ERs and induce rapid non-genomic effects by which they modulate cellular metabolism. Thus, they can change the protein kinase and lipid kinase cell signaling pathways [1]. It is believed that the activation of these signaling pathways by isoflavones causes some beneficial effects, in particular in the tissues that are not specific targets for the estrogens. At the circulatory system, the isoflavones induce vasodilation by increasing the production of nitric oxide (NO) after the activation of the endothelial NO^- synthase. At the central nervous system, they improve the cognitive function by affecting cell membrane permeability and altering the neuronal excitability. In the skeletal system, the isoflavones inhibit the tyrosine kinase causing changes in the alkaline phosphatase activity. On the other hand, they induce the apoptosis of the osteoclasts, suppress the formation of osteoclasts [34], and prevent the bone demineralization [35].

Also, isoflavones influence the activity of some of the enzymes involved in the metabolism of the sex steroid hormones. In this way they inhibit 5α-reductase (the enzyme responsible for the conversion of testosterone to 5α-dihydrotestosterone) and aromatase (involved in the conversion of testosterone to estradiol) in low concentrations, but they increase the aromatase activity at high concentrations. Isoflavones have an affinity for sex hormone-binding globulin (SHBG) and they induce its expression. Therefore, they affect the free-steroid hormone level in the systemic circulation. But these outcomes depend on many factors, including species, gender, and the hormonal status [35].

Xenoestrogens can modulate the enzyme activity of aromatase. Thus, they induce alterations in the metabolism of fats and carbohydrates through effects on ERα. The decrease of endogenous estrogen levels on ERα, aromatase inhibition or the existence of mutations affecting the enzyme activity has been correlated with visceral obesity or truncate, hyperlipidemia, glucose intolerance and insulin resistance, low physical activity, and reduced energy expenditure. Isoflavones compensate for the deficit of estrogens and have the ability to prevent the associated negative effects. Asian diets, rich in isoflavones, are correlated with low incidence of obesity and metabolic syndrome, favorable plasma profile, and a reduced body mass index in postmenopausal women [4].

4. Health benefits of isoflavones

4.1. Isoflavones and their effects on diseases

Numerous epidemiological and clinical studies have demonstrated the protective role of dietary isoflavones against development of specific menopause symptoms [36–38] and several chronic diseases, including cardiovascular diseases [39, 40], osteoporosis [38], cognitive impairment [37], and hormone-dependent cancers [41–43]. Based on human health benefits of soy diet, the Food and Drug Administration (FDA) approved the use of the following health

claim on the labels: "25 grams of soy protein a day, as part of a low in saturated fat and cholesterol, may reduce the risk of heart disease" [44].

Isoflavones, as all polyphenols, have a strong antioxidant activity. They can neutralize free radicals and prevent the lipid peroxydation by stopping the chain reactions. Also, isoflavones induce the antioxidant enzymes (glutathione peroxidase, catalase, and superoxide dismutase) and inhibit the expression of some enzymes, such as xanthine oxidase [1]. The antioxidant protective action of isoflavones from soy or plant extracts, such as *Trifolium pratense* L. or *Genista tinctoria* L., was proven in clinical studies [45, 46], as well as in animal models [32, 47].

4.2. Anticarcinogenic activity of isoflavones

The anticarcinogenic potential of isoflavones is based on multiple actions: binding to estrogen receptors (ERs), changing of cell signaling pathways, and inhibition of the key enzymes involved in the metabolism of sex hormones. Also, the anticarcinogenic potential of isoflavones has positive effects through independent mechanisms which do not involve ERs, such as antioxidant activity, reduction in the bioactivation of carcinogens, and stimulation of detoxification [2, 48].

Anticarcinogenic activity of genistein has been assessed more thoroughly among isoflavones. Genistein initiates apoptosis, alters cell proliferation and angiogenesis, and inhibits metastasis in many types of cancer cells [49]. It is a tyrosine kinase inhibitor. Therefore, in breast cancer cells, it slows down tumorigenesis; in the circulatory system, it prevents tumor vascularization; in the nervous system, it induces neuroprotective effects. In addition, genistein affects tumorigenesis by inhibiting DNA topoisomerases I and II [50], alteration of epigenetic regulations (both histone methylation and DNA methylation), and activating tumor suppressor genes [51]. As a polyphenol, genistein has antioxidant [1] and anti-inflammatory potential [52]. Another possible action pathway for genistein is the competitive inhibition of estrone metabolism through cytochrome P450 isoenzymes by altering the 2-hydroxy-estrone (2-OH-E_1)/16α-hydroxy-estrone (16α-OH-E_1) ratio, as noticed in vitro [53]. While 2-OH-E_1 is a weak estrogen, 16α-OH-E_1 has an important role in carcinogenesis, showing a strong estrogen effect and genotoxic properties [54]. 16α-OH-E_1 covalently binds to the estrogenic receptors and thus stimulates cell proliferation [55]. The ratio 2-OH-E_1/16α-OH-E_1 has been proposed and studied as a biomarker of breast cancer risk [55–59], but now its significance is controversial. In high concentrations, genistein decreases the hydroxylation of estrone in position 2 in favor of hydroxylation in position 16α [55]. Other studies show that genistein has no mutagenic or clastogenic activity in vivo. But in high concentration of genistein, it has clastogenic potential in vitro, explained by the topoisomerase inhibitory effect, which is known to cause chromosome damage above a certain threshold dose [60].

Anti-proliferative effects of high concentrations of genistein were demonstrated in all breast cancer cells, both ER positive and ER negative. However, there are several studies showing that genistein shows both anti-proliferative and proliferative effects, depending on the concentration, type of tumor, level of endogenous estrogens present in the tissue, or development stage. At low physiological concentrations, genistein stimulates tumorigenesis and cancels the

effects of tamoxifen in ER-positive breast cancer cells [50]. Similar dual effects were observed in the case of tamoxifen and other selective estrogen receptor modulators (SERMs) [16].

In fermented soybean products (e.g., natto, miso, tempeh), aglycons can suffer changes under the effect of enzymes produced by the microorganisms involved in the fermentation process. Thus, *ortho*-hydroxygenistein (6-OHG, 8-OHG, 3′-OHG) and *ortho*-hydroxydaidzein (6-OHD, 8-OHD, 3′-OHD) were identified. These compounds are not synthesized by the plants. The hydroxylation reaction that occurs in the *ortho* position gives molecules a high antioxidant potential and a free radical scavenging activity. Moreover, several of their abilities have been proven: to suppress cell proliferation and to inhibit tyrosinase (anti-melanogenesis properties) and antimutagenic, anti-inflammatory, and hepatoprotective properties [18].

Equol has a higher estrogenic potential than daidzein, its precursor, and a preferential affinity for ERβ, as it has already been stated. This detail is of high interest for its beneficial effect in the treatment of prostate cancer, since both isomers, *S*-(−)equol and *R*-(+)equol, can bind in vivo dihydrotestosterone without having an affinity for the androgen receptor. Therefore, equol prevents the endogenous hormone to exert its stimulating effect on prostate growth. In addition, equol possesses the highest antioxidant capacity of all isoflavones tested. It causes blood vessel relaxation and modifies the inflammatory response in activated macrophages and has beneficial effects in cardiovascular and inflammatory diseases [52].

4.3. Effects of isoflavones on hormone-dependent cancers

Clinical studies show contradictory results of the efficacy of isoflavones in the treatment of breast cancer. The effects depend on a number of factors such as age, gender, hormonal status, type of isoflavones consumed (soy proteins or isolated isoflavones), dose, diet (type of food), and extent of consumption [2].

A recent meta-analysis of 35 studies shows that soy isoflavones lower the risk of breast cancer in both premenopausal and post-menopausal women. The effect is more evident in Asian women than in those living in Western countries, probably due to differences in quality (traditionally fermented foods) and quantity of the isoflavone products ingested [41]. In Asian women, a diet rich in soy food lowers breast cancer risk with 30% [61]. A higher prevalence of equol-producer phenotype in Asian population can be an essential factor. Equol-producer phenotype is associated with a substantial reduction in the risk of breast cancer. Several specific biomarkers are favorable modified, such as sex hormone-binding globulin (SHBG) and steroid hormone levels in plasma, a higher urinary 2-hydroxy-estrone/16α-hydroxy-estrone ratio, and a lower mammographic breast density [2]. However, because several studies have provided mixed or contradictory results, the general recommendation for patients diagnosed with estrogen-dependent breast cancer is to avoid consuming high quantities of products containing isoflavone. Indeed, isoflavones are selective estrogen receptor modulators (SERMs), and their effects would depend on multiple factors.

Another meta-analysis of five cohort studies that included more than 11,000 female patients diagnosed with breast cancer focused on the post-diagnostic relationship between consumption of soy foods and mortality or cancer recurrence. The study concluded that the ingestion

of soy foods reduced mortality and recurrence in all types of breast cancer, especially in the ER-negative, ER-positive/PR-positive, and postmenopausal patients [42]. In women diagnosed with breast cancer under tamoxifen treatment, the consumption of plants containing isoflavones did not alter plasma levels of the drug and its metabolites [62]. Moreover, a recent study shows that a moderate intake of soy isoflavones (5–10 g soy protein/day) would have an optimal effect on tamoxifen treatment on these patients [63].

In some studies [64], excessive consumption of soy was associated with a negative impact on male fertility and reproductive hormones and the disruption of the thyroid gland function. In other studies these effects were inconsistent [65].

Isoflavones can modulate the toxicity of other xenoestrogens, but the interactions are complex and difficult to predict relying only on in vitro steroid receptor affinities [66]. In these kinds of interactions, multiple mechanisms are involved, both estrogen and non-estrogen type, such as oxidative stress [32, 47, 53]. European Food Safety Authority (EFSA) has recently conducted a systematic study of published medical literature, focusing on the correlation between the intake of soy isoflavones and the induced effects on the breast (mammographic density, proliferative marker Ki67 expression), uterus (endometrial thickness, histopathology changes), and thyroid (the thyroid hormone). Results showed that the intake of 35–150 mg isoflavones/day does not affect these organs in peri- and postmenopausal women [17]. Isoflavones have demonstrated prostate cancer efficacy in several studies: in vitro, on prostate cancer cell lines, in vivo, and in numerous clinical trials [43, 67, 68]. Conclusion of a recent meta-analysis suggests that phytoestrogen intake, mostly genistein and daidzein, can be correlated with a decreased risk of prostate cancer [69].

5. Recent advances in analytical methods of isoflavones

5.1. Isolation of isoflavones in foods and vegetable materials

In recent years, due to the health benefits provided by isoflavones, higher attention has been paid to the analytical methods that allow identification and quantification of isoflavones from different types of samples: (a) food, for dietary intake assessing [15, 70]; (b) food supplements, for standardization of nutraceuticals [5, 71]; (c) vegetable products, for phytotherapeutic evaluation [19, 20, 28]; and (d) human biological samples (plasma, urine) [5]. These analytical methods are commonly used for isoflavone bioavailability assessing and in pharmacokinetic or pharmacological studies.

Isoflavones are solubilized from food or vegetable material by refluxing or maceration, shaking, and stirring [72]. The isolation of isoflavones from the mixture can be achieved either by conventional methods, liquid-liquid extraction [11, 15, 19] or Soxhlet, or by modern ones—supercritical fluid extraction, ultrasound-assisted extraction [19, 71], pressurized fluid extraction, microwave-assisted extraction, and solid-phase extraction [5, 73] (**Table 4**).

The methods used to isolate isoflavones from food are selected function of the nature of the food, the type of the isoflavones analyzed (the total of aglycones or aglycones and

Analytes	Sample	Extraction method	Detection	Run time (min)	LOQ	References
3 IFs	Soy dry extract	Sonication/ Steam bath	HPLC-DAD	20	40–100 ng/mL	[71]
3 IFs, Cou*	10 plant species	UAE**	ULPC-PDA	4	1.97–4.08 ng/mL	[19]
12 IFs	Soybean seeds	Maceration	HPLC-UV	60	NA#	[70]
12 IFs	Soybeans, soy products	Maceration	HPLC-DAD	30	<600 nmol/L	[72]
3 IFs	Coffee	Refluxing	HPLC-DAD	35	13.7–25.0 ng/mL	[14]
17 IFs	Soymilk	Refluxing	LC-ESI(+)-MS/ MS	NA#	NA#	[74]
7 IFs, Cou*	2 plant extracts	Refluxing or Maceration	LC-ESI(−)-MS/ MS	18	40 ng/mL	[20]
5 IFs, Cou*	7 plant extracts	Maceration or percolation	ULPC- ESI(+)-MS/ MS	5.5	5–10.78 ng/mL	[28]
5 IFs	Legumes	SPE C18	UHLPC- ESI(+)-MS/ MS	18	0.1–1 ng/mL	[73]
5 IFs	Coffee	SPE C18	HPLC-ESI-MS/ MS	18	0.05–1 ng/mL	[75]

* Cou, coumestrol.
** UAE, ultrasound-assisted extraction.
# NA, not available.

Table 4. HPLC and UPLC methods applied for analysis of isoflavones in different samples.

glycosides), and the instrumental method used for identification and quantification. Several examples are presented below.

Liggins et al. isolated isoflavones from cereals and derivatives after a prior sonication in a polar solvent (methanol/water 4:1, v/v), in order to break apart the cellular material, followed by filtration and evaporation of the solvent under nitrogen. In order to determine the total aglycones, glycosides were hydrolyzed in an acid medium (0.1 M acetate buffer, pH 5) by overnight incubation at 37 °C in the presence of cellulase (enzyme used for hydrolytic removal of the hydrolysis resulted carbohydrates). Aglycones were extracted into ethyl acetate and were derivatized and analyzed using GC-MS [15]. Otieno et al. analyzed isoflavones from fermented and unfermented soy milk. For the solubilization of analytes, the freeze-dried sample was refluxed in methanol for 1 hour and filtered, and after adding the internal standard, the solvent has been evaporated to dryness under nitrogen. The residue has been suspended into a buffer (10 mm ammonium acetate containing 0.1% trifluoroacetic acid) and centrifuged, and the supernatant was filtered and analyzed using high-performance liquid chromatography (HPLC) [74].

Extraction and analysis of isoflavones in soybeans can be realized through maceration of the powdered beans with 70% ethanol at room temperature, for 24 hours under constant stirring.

After centrifugation and filtering, the supernatant is analyzed directly by HPLC [70]. Also, analysis of isoflavones contained in food supplements requires a simple preparation of the samples: fine powdering of tablets, refluxing in 80% methanol for 1 hour, filtering, and injection into the HPLC system [5].

Hydroalcoholic extracts or tinctures can be prepared from either fresh or dry and pulverized vegetable materials. The hydroalcoholic extracts can be made in 70% ethanol or methanol, by refluxing and filtration; by cold maceration, pressing, and filtration [20]; by percolation [28]; or using modern methods, such as ultrasound-assisted extraction in 50% ethanol [19]. The extracts can be analyzed directly by LC-MS/MS, after an adequate dilution [20], or they can be subjected to an acid hydrolysis [19] in order to release aglycones. Further, the aglycones can be assessed directly or after liquid-liquid extraction, for a concentration of the analytes [19].

In biological samples (e.g., plasma and human urine) isoflavones can be found in different forms: as aglycones (active metabolites), aglycone derivatives (with or without bioactivity), or conjugated metabolites (β-glucuronides and sulfate esters). Isoflavone analysis can focus on individual quantification of aglycones and their metabolites or quantification of aglycones after the hydrolysis of conjugated forms. Hydrolysis of conjugated metabolites is achieved by incubation at 37 °C with a mixture of β-glucuronidase/sulfatase in the presence of a buffer (0.5 M acetate) at pH 4.5 for several hours or overnight. Isolation of free forms and/or of those freed after hydrolysis can be done by liquid-liquid extraction or solid-phase extraction [5].

5.2. Quantification of isoflavones in foods and vegetable materials

For isoflavone identification, the following chromatographic methods are used: gas chromatography coupled with mass spectrometry (GC-MS) [5, 15], high-performance liquid chromatography (HPLC) with UV detector (photodiode array, PDA) [28, 70, 71], fluorescence detector (FLD), electrochemical detector (ECD) or mass spectrometer detector (MS) [20, 74, 75], and, less often, capillary electrophoresis (CE).

Quantification of isoflavones and their derivatives can be achieved in two ways: (a) by determining the free aglycons after a prior acid hydrolysis [19, 70, 72], alkaline hydrolysis [72], or enzymatic hydrolysis [72] of the glycosides in the sample and (b) by simultaneously analyzing the glycosides and aglycones present in the sample [20, 28]. GC-MS methods are used less lately, because they require an additional step of isoflavone derivatization to the volatile compounds [5, 15]. This additional step increases both the time and the cost of the analysis and represents a potential source of error [28].

Generally, HPLC-UV is not sensitive enough (**Table 4**) for the quantification of small levels of isoflavones from plant extracts [19] or human plasma [5]. This method often requires a hydrolysis step to transform glycosides into aglycones followed by the quantification of total aglycones from the sample [71].

In order to correctly identify new isoflavones or isoflavone derivatives present in the samples analyzed, liquid chromatography coupled with mass spectrometry (LC-MS) and tandem mass spectrometry (LC-MS/MS) are the preferred methods (**Table 4**), due to the advantages: speed, selectivity, sensitivity, and robustness. In addition, mass spectrometry detection allows sure

determination of the compounds based on molecular weight and ion charge. For the quantification of isoflavones, the pseudo-molecular ions or the ionic fragments resulted after fragmentation are monitored. In LC-MS/MS analysis, compound identification can be achieved even if their separation is not complete, and it is an advantage [74]. A shorter analysis can be realized by ultra-performance liquid chromatography (UPLC) [19, 28]. This method uses columns with very small size of the packing particles (1.7 μm) and consequently performs separations with superior resolution in a shorter time and a lower consumption of the mobile phase.

The isoflavones have polyphenolic structure and can easily lose a proton to form negative pseudo-molecular ions [M-H]- [20]. However, they can also be detected after ionization in positive mode to [M + H]+ [74]. Isoflavones are polar compounds and they form ions in solution. For these type of compounds, electro-spray ionization (ESI) is the most commonly used source to obtain analytical ions. Atmospheric pressure chemical ionization (APCI) is the source preferred for non-polar analytes that ionize in the gas phase. The isoflavones often give poor response in this ionization source [28]. The fragmentation patterns of isoflavone glycosides (malonyl-glycosides, acetyl-glycosides, glycosides, aglycones) follow a similar trend. However each compound has a unique fragmentation pattern that allows their accurate identification (**Table 5**) [74].

Isoflavone	[M + H]+	Transitions	[M − H]-	Tranzitions
Daidzein	255 [28, 74]	→199 [28]	253 [20, 73, 75]	→208, 132 [73, 75]
Formononetin	269 [28]	→197 [28]	267 [20, 75]	→252, 223 [75]
Genistein	271 [28, 73, 74]	→153 [28]	269 [20, 73, 75]	→159, 133 [73, 75]
Biochanin A			283 [73, 75]	→268, 239 [73, 75]
Glycitein	285 [74]	→270, 257, 229, 196, 166 [74]	283 [20]	
Daidzin	417 [28, 73, 74]	→255 [28, 73, 74], 199 [73]	415 [20]	→253 [20]
Ononin			429 [20]	→267 [20]
Genistin	433 [28, 74, 75]	→271 [28, 73–75], 91 [73, 75]	431 [20]	→268, 269 [20]
Glycitin	447 [74]	→428, 285 [74]		
Ac-daidzin	459 [74]	→441, 255 [74]		
Ac-genistin	475 [74]	→431, 271 [74]		
Ac-glycitin	489 [74]	→471, 285 [74]		
Mal-daidzin	503 [74]	→485, 255 [74]		
Mal-genistin	519 [74]	→501, 271 [74]		

Table 5. Ions (*m/z*) and transitions monitored for isoflavone quantification.

6. Conclusion

Dietary intake of isoflavones is widespread, mainly due to the high consumption of soybean products. Health benefits of isoflavones justify the interest for this class of bioactive compounds, but the controversial outcomes of some clinical and epidemiological studies require further investigations. In the context of these researches, the analytical methods applied for assessment of isoflavones are very valuable. They allow for the evaluation of dietary intake of isoflavones, equating the health benefits and the circumstances in which they are exerted, and highlight the natural sources of isoflavones with phytotherapeutic potential.

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