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CHROMATOGRAPHIC QUANTIFICATION OF ISOFLAVONES (WHY AND HOW)

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SUMMARY

Recent advances in chromatographic analysis of isoflavones, which can reach physiologically significant concentrations in human body fluids as a result of nutrition with food containing soy products or diet supplementation with phytoestrogen preparations, are surveyed and evaluated. Soy-derived food products and nutraceuticals are claimed to be beneficial for cardiovascular health and bone metabolism and to contribute signifycantly to cancer chemoprevention. Because clinical trials involving plant-derived isoflavone-rich materials have been rather inconclusive, it seems important to discuss analytical means by which pharmacokinetic and pharmacodynamic data are generated.

Alphabetical List of Abbreviations

ACN – acetonitrile, API MS – atmospheric-pressure ionization mass spectrometry, APCI – atmospheric-pressure chemical ionization, BI – biochanine A, t-BDMS – *tert*-butyldimethylsilyl, BIGL – sissotrin, CEAD – coulometric electrode array detector, CI – chemical ionization, C-IR MS – combustion-isotope-ratio mass spectrometry,CO – coumestrol, DA – daidzein, DAD – diode-array detector, DAGL – daidzin, DHB – dihydrobiochanin A, DHD – dihydrodaidzein, DHF – dihydroformononetin, ELISA – enzyme-linked immunosorbent assay, EC – electrochemical detector, EI-MS – electron impact mass spectrometry, EQ – equol, ESI – electrospray ionization, FAB – fast-atom bombardment, FDA – Food and Drug Administration, FO – formononetin, FOGL – ononin, GC – gas chromatography, GCP – good clinical practice, GE – genistein, GEGL – genistin, GLP – good laboratory practice, GY – glycitein, HPLC – high-performance liquid chromatography, ID-GC–MS-SIM – isotope dilution gas chromatography–mass spectrometry in selected ion monitoring mode, LC – liquid chromatography, LOD – limit of detection, LOQ – limit of quantifycation, LSI-MS – liquid secondary ion mass spectrometry, MALDI–TOF-

MS – matrix-assisted laser-desorption ionization time-of-flight mass spectrometry, MeOH – methanol, MS – mass spectrometry, MTBE – methyl *tert*-butyl ether, ODMA – *O*-desmethylangolensin, PDA – photodiode-array detector, RP – reversed phase, RIA – radioimmunoassay, SPE – solid-phase extraction, SIM – selected-ion monitoring, TFA – trifluoroacetic acid, TLC – thin-layer chromatography, TSP–MS – thermospray mass spectrometry, TR-FIA – time-resolved fluorescence immunoassay

INTRODUCTION

Some groups of natural products (e.g. flavonoids) are, because of their widespread occurrence and pleiotropic biological activity, of interest not only as phytochemical objects but also as agents influencing human health through nutrition, by supplementation of diet and prospectively, as medicinal compounds. Consequently, there is steadily growing demand for selective, sensitive and validated methods for their analysis and quantification, to be applied in agriculture, the food industry, herbal medicine, and clinical studies.

Flavonoids constitute a large (estimated as several thousand entities) group of secondary plant metabolites, abundant in fruits and vegetables, therefore important for human nutrition, although their aromatic and polyphenolic character places them outside the nutrient category. It should be kept in mind that nutritional sciences are not limited to calorie counting – in their present, rational version they are recognized as an important aspect of healthcare, taking into account all constituents, e.g. fiber, antioxidants, phytoestrogens etc. [1–4]. Obviously, despite their natural origin and wide acceptance, on the basis of traditional use, all non-nutritional constituents of food deserve, at least scientifically, attention similar to that devoted to other xenobiotics, e.g. synthetic drugs. This view, supported by a plethora of recent studies, which indicate a multitude of molecular targets, and consequently different biological action, of plant flavonoids [5–11] calls for specification of the variety plant raw materials and monitoring of their individual constituents from human intake to excretion; this in turn creates considerable problems in both qualitative and quantitative analysis.

To discuss the scope of the problem we have decided to focus in this review on isoflavones, a well defined, but smaller by an order of magnitude, sub-class of plant flavonoids; their typical skeletons, A–H, are shown in Fig. 1. Several arguments can be used as a rationale for this decision. Unlike other flavonoids, which are widespread in the plant kingdom, oc-

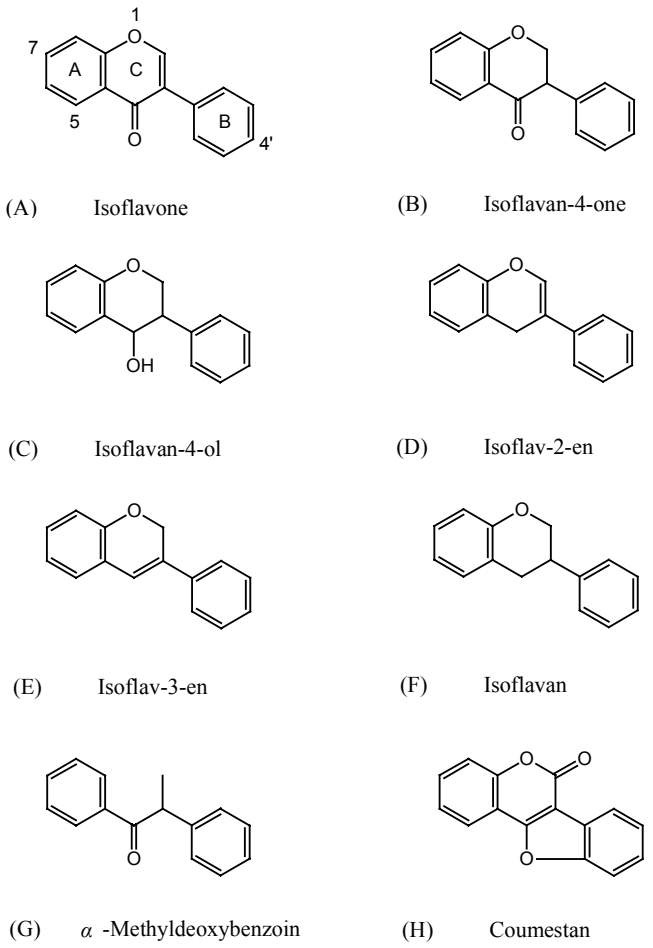


Fig. 1

Types of isoflavone structure

currence of isoflavones is practically limited to legumes (*Leguminaceae* family; the names *Fabaceae* and *Papilionaceae* are also used). Although this family provides a great variety of edible crops (e.g. beans and peas), only one plant, soy (*Glycine max,* Merrill), contributes nutritionally to significant isoflavone intake by humans. Recently a few more isoflavone-containing plants (classified as herbs, rather then food) have been added, in view of the growing consumption of nutraceuticals, taken without prescription, for variety of ailments, or as dietary supplements, for chemoprevention. Thus we have clearly defined botanical and nutritional sources of the

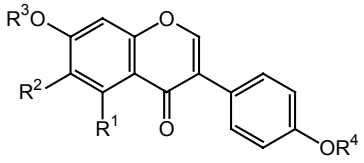
substances which we aim to study as analytes, and their chemical characteristics are usually also well known from the outset of the investigation.

Isoflavones are widely believed to have beneficial effects on human health. This view stems mainly from epidemiological data, indicating an inverse correlation between levels of soy-derived food consumption and the incidence of certain types of cancer. Similar data support the general belief that soy consumption at the level characteristic of Asian society prevents cardiovascular ailments and post-menopausal effects, including osteoporosis [7–10]. Surprisingly, numerous clinical trials have failed to produce convincing evidence in favor of opinions based on epidemiological observations. This apparent discrepancy cannot be resolved without well designed, meticulous studies of isoflavone pharmacokinetics and pharmacodynamics, which in turn require sensitive, selective and reproducible analytical methods. Although identification and determination of isoflavones was achieved a long time ago, as part of traditional phytochemical analysis [12], the most recent achievements in separation and detection technology are needed to set up procedures suitable for clinical analysis and which can account for all the isoflavone sample administered and answer fundamental question about its biodistribution, metabolism, and excretion. The paper reviews the wide background of isoflavone analysis but focuses on methods which, by virtue of low detection and quantification limits, can be of use in answering basic questions about nutritional safety and prospective medicinal applications of natural and chemically modified isoflavones [13–20].

STRUCTURAL DIVERSITY OF ISOFLAVONE ANALYTES

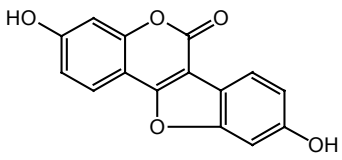
Because isoflavones have a variety of biological actions important to human physiology (e.g. estrogenic, inhibiting protein tyrosine kinases, and modulating activity of many other enzymes, as well as being antioxidants on molecular and cellular levels) [3–10], their specification in herbs, agricultural raw materials, food and nutraceuticals is of considerable importance. Soybeans, the main renewable source of isoflavones, contain, depending on the particular cultivar and subject to soil and climatic conditions, 0.1–0.3 mg g^{-1} of these secondary metabolites in fresh biomass at harvest. Considering the global production of soy, which exceeds 150×10^6 tons annually, hundreds of tons of isoflavones are consumed by cattle, livestock, and the human population. Obviously, the medical effects of this massive challenge should be recognized and further monitored. Soybeans and other

Leguminaceae plants are known to contain genistein (GE), daidzein (DA) formononetin (FO), glycytein (GY), biochanine A (BI), and coumestrol (CO) (Fig. 2), but frequently only the first two, the main constituents, are determined quantitatively.



	R^1	R^2	R^3	R^4
Genistein (GE)	OH	H	H	H
Genistin (GEGL)	OH	H	Glu	H
Daidzein (DA)	H	H	H	H
Daidzin (DAGL)	H	H	Glu	H
Biochanin A (BI)	OH	H	H	CH_3
Sissotrin (BIGL)	OH	H	Glu	CH_3
Formononetin (FO)	H	H	H	CH_3
Ononin (FOGL)	H	H	Glu	CH_3
Glycitein (GY)	H	OCH_3	H	H
Glycitin (GYGL)	H	OCH_3	Glu	H

Glu = β-D-glucopyranosyl



Coumestrol (CO)

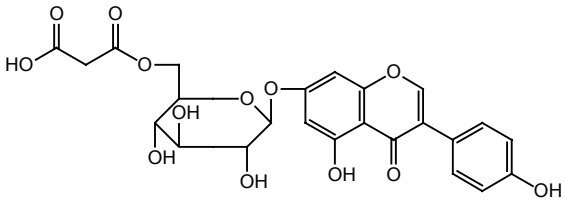
Fig. 2

Isoflavone aglycones and their glucosides

Relatively recently it has been realized that all these compounds occur in the native biological state, in plant tissue, in the glycosylated form. The most common glycosylation pattern is involvement of the 7-*O*-phenolic group in a β-D-glucopyranosidic bond (Fig. 2). Glycosides corresponding to the aglycones listed above are known in phytochemistry and pharmacognosy under individual names, e.g. genistin (GEGL), daidzin (DAGL), sissotrin (BIGL) and ononin (FOGL). Several other isoflavone glycosides, involving different sugar moieties (rhamnose, xylose, apiose), have been isolated from *Leguminaceae* plants, but are presently of lesser concern with regard to their effect on human health.

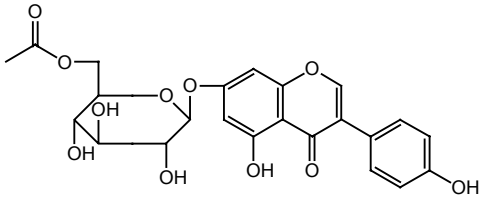
Isoflavones are secondary metabolites of higher plants arising from the malonate–shikimate biosynthetic pathway, which produces great variety of phenylpropanoid aglycones. Two kinds of post-translational modification are characteristic of this group of natural products – *O*- (and occasionally *C*-) glycosylation and esterification by biogenic carboxylic acids. Accordingly, the full specification of isoflavone constituents present in soy raw materials and soy-derived foodstuff, involves, in addition to the compounds already mentioned (Fig. 2), also glycosides and esterified glycosides bearing 6′-malonyl or 6′-acetyl residues (Fig. 3). The analytical distinction between glycosides and aglycones is quite important, because the physicochemical properties, stability, bioavailability, and pharmacodynamics of the two classes are completely different. It is generally assumed that glycosides are devoid of the biological activity characteristic of the corresponding aglycones [7,9,17,22].

Natural isoflavones are extensively metabolized in the human body by enzymatic processes characteristic of the host physiology, and also by intestinal bacteria. Plant glycosides are first deglucosylated and the aglycones are conjugated to glucuronic acid and/or sulfate to form soluble derivatives easily excreted by the renal system (Fig. 4). Alternatively, aglycones are involved in a sequence of processes, starting with *O*-demethylation and followed by reduction, which ultimately leads to degradation of the isoflavone skeleton, producing simple aromatic compounds, for example carboxylic acids and alkylphenols. Thus, the final products of genistein biodegradation are 4-ethylphenol and 4-hydroxypropionic acid. Some typical degradation pathways, starting from saturation of the chromenone C–C double bond, are presented in Fig. 4. This particular transformation of daidzein is important pharmacologically, because it affords a metabolite, equol, an isoflavan, which is known to be more estrogenic than any native constituent of soy. When considering the structural diversity of the

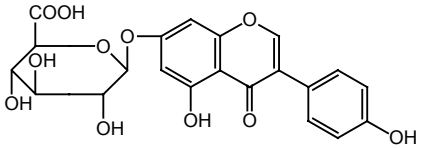


genistin 6"-O-malonate

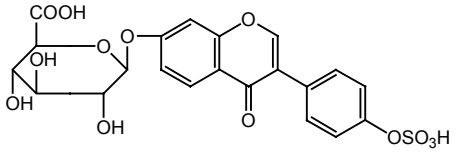
$-CO_2$



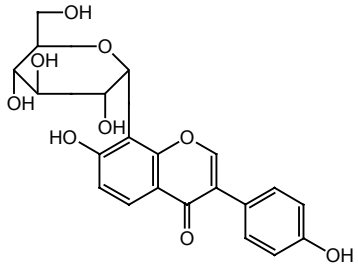
genistin 6"-O-acetate



genistein 7-O-β-D-glucuronate



genistein 7-O-β-D-glucuronate-4'-O-sulphate



8-C-α-D-glucopyranosyldaidzein (puerarin)

Fig. 3

Acylated glycosides, glucuronides, sulfates, and C-glycoside

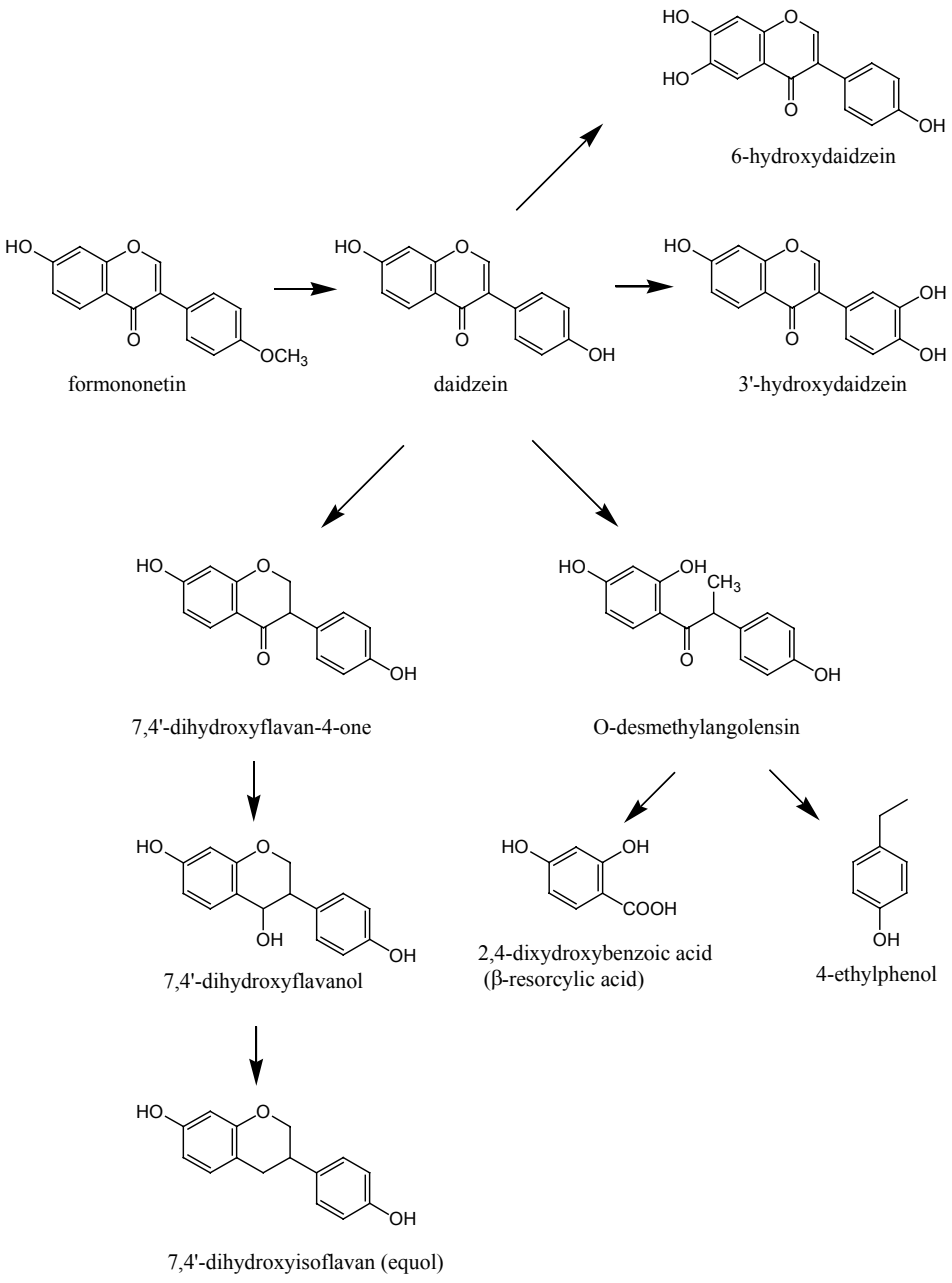


Fig. 4

Typical metabolic pathways of isoflavone aglycones

native isoflavones occurring in plants, a variety of hydroxylation and *O*-methylation patterns must also be taken into account, and the presence of sugar moieties other than glucose (e.g. rhamnose, xylose, apiose, etc.). Skeletal modifications are also encountered, C-prenylated isoflavones being typical examples. It must be remembered that every individual plant-derived isoflavone is likely to be subject to oxidative and/or reductive metabolism and every metabolite, in turn, is a likely substrate for conjugation enzymes. Therefore complete analysis of a sample of plant origin, metabolized by a mammal with physiological intestinal bacterial flora can easily involve tens of individual compounds with a considerable range of structural and physicochemical variety.

Such multitude of different analytes cannot be easily quantified by use of only one separation and detection procedure. Because of continual calls for efficient analytical systems capable of accommodating a wide array of structures in pharmacokinetic analysis, it seems compelling to summarize and critically evaluate recent technical achievements in isoflavone analysis suitable for studies of intake, biodistribution, and metabolism [21–30] (Tables I and II).

BRIEF SURVEY OF REQUIREMENTS FOR SPECIFICATION OF ISOFLAVONE-CONTAINING MATERIALS

Isoflavone-containing soybeans are among the most important agricultural crops, a traditional source of food in Asian countries, and an important source of food additives and modifiers used throughout the world (e.g. in the manufacture of meat products). Because isoflavones are clearly estrogenic, among other distinct biological activity, and tons of them end up in food and animal feed, they should be traced as a matter of basic toxicological and environmental protection. Two principal products of soy processing are soy oil and soy protein; the latter usually contains all the isoflavones and their glycosides. When the soy industry was first established, the astringency and bitter taste of non-protein constituents of soy was perceived as an obstacle and processes for extraction of isoflavones were designed, providing purified soy protein (soy flour) [2]. The technology currently used preserves isoflavones in the protein fraction (one of the most important raw materials in the entire food industry), in acknowledgment of the health claim [2,9,10,17,28], accepted by the FDA, of the beneficial effect of soy on the cardiovascular system. Obviously, all soy raw materials and soy-containing food products should be specified for

Table I

Determination of isoflavones in mammalian biological fluids

№	Analyte/composition	Sample/matrix	Separation method	Detection method	Remarks	Ref.
1	Formononetin and Biochanin A	Human urine	GC	MS	New reduced metabolites of formononetin (DHF and angolesin) and biochanin A (DHB and dihydroxyangolesin) were identified in urine samples. Possible metabolic pathways are presented.	[31]
2	Daidzein and Genistein	Human urine and soybean food	Gradient HPLC on C_{18} after accelerated solvent extraction (for soybean products)	EC with carbon paste electrode-coulometer LOD: DA: 480 pg mL^{-1} GE: 394 pg mL^{-1}	A highly sensitive HPLC method with electrochemical detection and carbon paste electrode (adsorptive transfer stripping square wave voltammetry) was developed for determination of isoflavones.	[32]
3	Genistein and its new derivatives	Human plasma and culture media	RP-HPLC after liquid–liquid extraction	UV, LOD: GE 0.0037 μM Deriv. 0.0008–0.0052 μM LOQ: GE 0.037 μM Deriv. 0.0021–0.0230 μM	Specific, sensitive and technically simple method was used to evaluate concentration levels of investigated compounds with cytotoxic activity in experiments with HL-60 (human leukemia) cell line.	[33]
4	Isoflavone metabolites	Serum of mice fed soy proteins	HPLC	ESI-MS–MS LOD: 0.018 mμM GE 0.035 mμM DHD, LOQ: 0.04 mμM GE 0.08 mμM DHD	The study indicated the presence of DHD in serum of mice fed diets containing soy proteins.	[34]
5	Genistein, Daidzein, Biochanin A, Coumesterol	Rat plasma	Gradient HPLC after rapid on-line extraction on SPE	ESI-MS–MS LOD: 100 pg mL^{-1}, LOQ: 1 ng mL^{-1}	In the study the "n-in-one" protocol was used. The methodology is the key point of the paper. Each plasma sample analysis took less than 2 min. Phytoestrogens have been reported to circulate in soy-formula fed infants at concentrations that are 13000–22000 times higher than plasma estrogen concentrations, which range from 40–80 pg mL^{-1} in early life.	[35]
6	Genistein, Daidzein, Equol, Formononetin, Biochanin A	Bovine milk	HPLC	ESI-MS–MS	The developed methodology was applied to various milk samples, and the occurrence of isoflavones was demonstrated in the concentration range 1–30 ng mL^{-1}. The method designed to asses a global risk from phytoestrogens.	[36]

№	Analyte/composition	Sample/matrix	Separation method	Detection method	Remarks	Ref.
7	Genistein, Daidzein, Equol, *O*-Desmethyl-angolesin, Dihydrogenistein, Dihydrodaidzein	Human urine and rat urine	Isotope dilution GC after enzymatic hydrolysis, extraction into ethyl ether and purification by several types of ion-exchange chromatography	MS LOD: 2 nmol L^{-1}	The study demonstrated that human intestinal flora is necessary to produce daidzein metabolites (EQ, ODMA), but not for hydrolysis and absorption of the isoflavone glucosides.	[27]
8	Daidzein, *O*-Desmethyl-angolesin, Equol, Genistein, Glycitein	Urine	GC after SPE and derivatization	MS, LOD: For DE – 1.2 ng mL^{-1} For EQ – 5.3 ng mL^{-1}	The authors reported an assay for phytoestrogens which is sensitive, accurate and uses 0.2 mL of a sample. The method is suitable for epidemiological studies and consists of a simple preparation procedure on single SPE and derivatization for GC–MS analysis.	[37]
9	Daidzein, *O*-Desmethyl-angolesin, Equol, Genistein, Glycitein	Serum	HPLC after SPE	ESI-MS–MS LOD: 10 pg mL^{-1} except EQ, for which 100 pg mL^{-1}	A simple, highly automated sample preparation procedure requiring only 0.2 mL of sample and utilizing one SPE stage was reported. The method is suitable for measuring concentrations of phytoestrogens in blood samples collected from large epidemiological studies.	[38]
10	Daidzein and Genistein	Soy nuts, human serum and urine	Soy nuts: RP-HPLC after extraction into 80% methanol by refluxing and then filtration Plasma: GC after SPE on C_{18}, enzymatic hydrolysis and derivatization with *tert*-butyldimethylsilyl ether Urine: HPLC on C_{18} after hydrolysis without derivatization	UV or ESI-MS for RP-HPLC MS for GC	Attention is drawn to the fact, that there is a tendency in clinical trails to use relatively large dietary intakes of soy isoflavones, far exceeding typical intakes for people living in Asia.	[39]
11	Daidzein and Genistein, Genistin, Daidzin	Human plasma and urine	HPLC on C_{18} after extraction and enzymatic hydrolysis with H-2 β-glucuronidase/sulfatase or B-3 β-glucuronidase	PDA or UV	Authors demonstrated that GE, DAGU and GEGU are not toxic to NK (natural killer) cells at physiological concentrations. The glucuronide forms are more active than the genistein in activating NK cells.	[40]

№	Analyte/com-position	Sample/ matrix	Separation method	Detection method	Remarks	Ref.
12	Daidzein and Genistein	Human plasma	HPLC on C_{18} after enzymatic hydrolysis	EC LOQ: 0.01 μmol L^{-1}	Suggestion was made that pharmacokinetics of GE and DA may strongly depend on a race and dietary habits (as consequence of differences in colonic microfloral populations).	[41]
13	Daidzein, Glycitein, Genistin	Soybean Human urine	Gradient HPLC, Mobile phase: A: 0.1% acetic acid in water, B: 0.1% acetic acid in ACN; Flow rate: 1.0 mL min^{-1} CE	UV–visible detection at 254 nm, LOD: 0.0005 mg g^{-1} 2D maps	Method of efficient extraction of isoflavones is described. Comparison of various analytical methods is made.	[42]
14	Daidzein, Equol, Genistein	Human breast tissue, urine and serum	HPLC; column: XterraMS C_{18}; flow rate: 1.5 mL min^{-1} mobile phase: A: 0.05% formic acid in water, B: acetonitrile–methanol 20:80 *v*/*v*; isocratic method	UV, LOD: 24–148 nmol L^{-1}, LOQ: 62.5–125 nmol L^{-1}	Procedures for enzymatic hydrolysis and extractions are described affording fast and reliable method of isoflavone determination	[20]
15	Equol, Daidzein, Dihydrodaidzein, Genistein	Blood, urine	HPLC	HPLC–MS, LOD: 40 nmol L^{-1}	Assay of endogenous hormones and isoflavones in blood and urine of postmenopausal women	[43]
16	Daidzein, Genistein, Equol, *O*-Desmethyl-angolesin		C_{18} HPLC after enzymatic hydrolysis and SPE on C_{18} cartridges	UV, LOQ: DA 1.3 ng mL^{-1} GE 2.4 ng mL^{-1} EQ 151 ng mL^{-1} ODMA 201 ng mL^{-1}	High level of recoveries of isoflavones (ca. 100%) were attained	[26]
17	Isoflavone aglycones	Human plasma, urine and feces	RP-HPLC after enzymatic hydrolysis and SPE on C_{18}	UV, LOD: 120 ng mL^{-1}	Recoveries ranged from 60 to 85% (plasma, urine and feces)	[26]
18	Isoflavone aglycones	Plasma, urine and tissue	RP-HPLC after enzymatic hydrolysis by glucuronida-se/sulfatase and liquid–liquid extraction	EC–Coulometer, LOD: 1–2 ng mL^{-1}	Recoveries of 85–95%	[26]

№	Analyte/composition	Sample/matrix	Separation method	Detection method	Remarks	Ref.
19	Equol, Daidzein,	Bovine plasma and urine	HPLC on C_{18} after enzymatic hydrolysis by glucuronidase/sulfatase of isoflavone metabolites	UV, LOD: In 1 mL of blood: 0.4 ng of DA, 13 ng of EQ In 1 mL of urine: 130 ng of DA; 4000 ng of EQ	Recovery: 73% EQ and 91% DA (from urine), 105% EQ and 92% DA (from plasma).	[26]
20	Genistein	Rat serum and tissues	C_{18} HPLC after enzymatic hydrolysis by glucuronidase/sulfatase and SPE	ES/MS and MS–MS with use of deuterated Genistein as internal standard, LOD: For tissues: ES/MS 0.04–0.09 pmol mg^{-1} (10–20 pg mg^{-1}) MS–MS 0.01–0.03 pmol mg^{-1} (3–8 pg mg^{-1}), LOQ: For serum: ES/MS 0.02 μmol L^{-1} MS–MS 0.005 μmol L^{-1}	Pharmacokinetic analysis of serum genistein showed a significant difference in the elimination half-life and area under the concentration–time curve between male and female rats. Endocrine-responsive tissues including brain, liver, mammary, ovary, prostate, testis, thyroid, and uterus showed significant dose-dependent increases in total genistein concentration. Female liver for example contained the highest amount of genistein (7.3 pmol mg^{-1}) whereas male liver only 0.67 pmol mg^{-1}).	[26]
21	Isoflavones	Human urine	Immunoassay after enzymatic hydrolysis by glucuronidase/sulfatase	Fluorescence, LOD: 0.1 ng mL^{-1}	Method suitable for the major soybean isoflavones (GE and DA).	[26]
22	Daidzein, Genistein, Equol, *O*-Desmethyl-angolesin	Human urine	GC after purification by ion-exchange chromatography, enzymatic hydrolysis and trimethylsilylation	MS, LOD: 0.04–0.08 nmol mL^{-1} for all compounds	Urinary isoflavone excretion accepted as a biomarker of both recent and past soy food intake in young-to middle-aged women who have relatively low and infrequent intake of soy foods (low concentration of isoflavone in blood). Isoflavone measurement in one overnight urine collection can provide information on current and/or usual consumption of soy foods in such populations and can distinguish, on average, between soy consumers and nonconsumers.	[44]
23	Genistein, Daidzenin and Glycitein	Human plasma and urine	HPLC on phenyl-hexyl column after enzymatic hydrolysis and extraction into MTBE for conjugated isoflavones and without hydrolysis for free isoflavones.	UV, LOD: Plasma/urine: GE 0.008/0.08 μmol L^{-1}, DA 0.007/0.09 μmol L^{-1}, GY 0.009/0.08 μmol L^{-1}	In the study minimal clinical toxicity was observed even at single doses that exceeded normal dietary intakes many fold. It was found that GE and DA were rapidly cleared from plasma and should not result in progressive accumulation of these isoflavones.	[45]

№	Analyte/composition	Sample/matrix	Separation method	Detection method	Remarks	Ref.
24	Daidzein, Genistein, Glycitein	Human plasma and urine	Gradient HPLC on C_8 after: extraction with MTBE (for free isoflavones in plasma) or SPE on C_{18} (for free isoflavones in urine) and enzymatic hydrolysis and extraction with MTBE (for plasma and urinary total isoflavones).	UV, LOQ: Free in plasma/urine: GE 6.3/14 nmol L^{-1} DA 12/2.2 nmol L^{-1}	Dietary supplements of purified unconjugated isoflavones administrated to humans in single doses exceeding normal dietary intake manifold resulted in minimal clinical toxicity. GE and DA (free and total) were rapidly cleared from plasma and excreted mainly in urine.	[29]
25	Genistein	Rat plasma Soya infant milk formula	For rat plasma: HPLC on C_{18} after SPE on C_{18} For soya milk: HPLC on C_{18} after extraction, enzymatic hydrolysis	ESI-MS–MS, LOD: 1 ng mL^{-1} for GE and genistein glucuronide, LOQ: 20 pg mL^{-1} for GE and genistein glucuronide	LC linked to tandem MS enables separation of polar metabolites from parent compounds without recourse to hydrolysis of conjugates or analyte derivatisation. Pharmacokinetic parameters: C_{max}, AUC and T_{max} for GE were similar to those reported in humans, which supports the use of the rat model for GE toxicity studies.	[30]
26	Daidzein and its metabolites	Rat serum and tissues, milk	For serum: RP-HPLC after extraction with hexane, enzymatic hydrolysis and extraction into diethyl ether For tissues: RP-HPLC after hydrolysis by proteinase K, SPE on C_{18}, hydrolysis by β-glucuronidase/sulfatase and extraction into diethyl ether	APCI-MS–MS, LOD: 5 nM	DA was investigated for its potential to alter fertility and to cause developmental toxicity to the reproductive tract in female rats. It was concluded, that supraphysiological concentrations of DA administered via the diet did not cause significant toxicity to the female reproductive tract or provide a protective effect against chemically induced mammary cancer in rats.	[46]
27	Genistein, Genistin, Daidzein, Daidzin, Glycitein, Glycitein glucoside, Dihydrogeistein, Dihydrodaidzein, Equol, *O*-Desmethylangolesin	Human plasma and urine	Gradient HPLC on C_{18} after enzymatic hydrolysis with sulfatase/glucuronidase and SPE	PDA and fluorimeter	The study demonstrated that enzyme hydrolysis of a purified, concentrated extract of isoflavones does not enhance the absorption of isoflavones in postmenopausal women.	[47]

№	Analyte/composition	Sample/matrix	Separation method	Detection method	Remarks	Ref.
28	Daidzein, Genistein	Human plasma and urine	Gradient RP-HPLC after enzymatic hydrolysis and extraction into ethyl ether	MS	The study suggested that there are significant differences in pharmacokinetic of sulfate and glucuronide conjugates of isoflavones. Potential implications of this finding for a metabolite bioactivity required to elicit potential health benefits are discussed.	[48]
29	Isoflavones	Mice plasma	Gradient HPLC on C_{18} after enzymatic hydrolysis and deproteinated with methanol	PDA	Male mice were fed a soy protein or casein diet supplemented with isoflavones and feces and plasma samples were analyzed to investigate the capacity to produce EQ from DA. The study indicates that the soy protein diet supplemented with isoflavone has an impact on the consumption and metabolism of intestinal microflora. It suggests that soy protein plays some roles in the effect of dietary isoflavones on the host through their effects on the intestinal microflora.	[49]
30	Formononetin, Biochanin A, Daidzein, Genistein	Human urine	Gradient HPLC on C_{18} after enzymatic hydrolysis, SPE on C_{18}	UV or EC or MS, LOD: 5 ng mL^{-1} for each isoflavone	Soybean isoflavone glycosides (GEGL and DAGL) and red clover isoflavone aglycones (FO, BI, GE and DA) were incorporated into a diet of 14 subjects for 2 weeks. The 24-h excretions of isoflavones in urine were measured. Although inter-individual variability was high, there was less intra-individual variability; the amounts excreted when subjects consumed the two sources of isoflavone were correlated ($r = 0.69$; $P = 0.007$). Subjects who excreted more isoflavone with one mix did so also with the other. This suggests that bioavailability and biological action may vary among individuals but appears to be consistent for an individual.	[50]

№	Analyte/composition	Sample/matrix	Separation method	Detection method	Remarks	Ref.
31	Isoflavones: Genistein, Daidzein Glycitein, Formononetin Metabolites: Dihydrogenistein, Dihydrodaidzein, *O*-Desmethyl-angolensin	Human plasma, serum and urine	LC, column: HydroBond PS (100 mm × 3.0 mm, 5 μm) C_{18} RP, HydroBond PS (25 mm × 3.2 mm, 5 μm) guard column, flow rate 0.25 mL min^{-1}; mobile phases: A: methanol–acetonitrile–0.5 acetic acid aq; B: methanol–acetonitrile–water; gradient method	PDA-ESI-MS detection: UV detection at 220–400 nM followed by ESI-MS at 260°C, LOQ: for urine 1–39 nM for plasma: 1–29 nM	Method validation conducted.	[19]
32	Genistein, Daidzein, Equol	Human and animal serum	HPLC: column: Ultracarb ODS (150 mm × 2 mm 3 μm); flow rate: 0.2 mL min^{-1}; mobile phase: acetonitrile – 0.1% acetic acid aq 35:65 (*v*/*v*),	UV, ES-MS deuterated internal standards were used, LOD: 0.01–0.001 μM, LOQ: 0.03–0.005 μM	SPE and enzymatic hydrolysis applied. HPLC method validated.	[18]
33	Daidzein, Genistein and their glucuronides, sulfates and sulfoglucuronides	Human urine	LC	Isotope dilution ESI-MS–MS, LOD: < 50 ng mL^{-1}	^{13}C Labeled compounds were synthesized and used as internal standards; sensitivity and precision of the method were determined.	[51]
34	Daidzein, Genistein, Formononetin, Biochanin A, Glycitin, Genistin, Daidzin	Human plasma and urine	Unconjugated isoflavones: GC on DB-1 fused silica column after group separation from their glucuronides and sulfates, SPE on C_{18} and derivatization with t-BDMS. Total isoflavones: GC after extraction and enzymatic hydrolysis, SPE on C_{18}, filtration through TEAP-LH-20 and derivatization with t-BDMS ether. Isoflavone supplements: C_{18} HPLC after extraction by refluxing in 80% methanol and filtration	MS for unconjugated isoflavones ESI-MS or UV for isoflavone supplements	The study shown a dose-dependent effect of isoflavones on reducing LDL cholesterol concentrations. It appeared that the presence of protein matrix is necessary for the effectiveness of isoflavones for lowering cholesterol level. Comments on complexity of nutraceutical compositions and its consequences are included.	[28]

№	Analyte/composition	Sample/matrix	Separation method	Detection method	Remarks	Ref.
35	Daidzein, Equol and Genistein	Human plasma, urine and feces	Gradient HPLC on C_{18} after enzymatic hydrolysis	UV	The results from the study indicate urinary recovery of DA and GE on the level about 25% of the given dose and only a few percentage of the total isoflavone dose was recovered in feces, probably due to bacterial breakdown of these compounds. Isoflavone bioavailability may not be affected by choice of background diet or food source of isoflavone aglycones.	[52]
36	Daidzein, Genistein, Daidzin and Genistin	Human plasma	For DA and GE: HPLC on C_{18} after extraction from plasma; For DAGL and GEGL: HPLC on C_{18} after enzymatic hydrolysis and extraction from biological samples	EC	The authors tested absorption of DA, GE, DAGL and GEGL in men after single and continuous intake. The result revealed the superior absorption of DA and GE in humans. It indicated that aglycones are more useful in maintaining a high level of isoflavone concentration in plasma than corresponding conjugates	[53]
37	Daidzein, Genistein, Daidzin and Genistin	Rat plasma	HPLC on C_{18} after enzymatic hydrolysis, extraction, centrifugation and dilution of supernatant	EC with amperometer	Absorption of isoflavone aglycones and glucosides was compared in rats. DA, GE, DAGL and GEGL were orally administrated and next their metabolite concentration in plasma was monitored for 30 min. After the glucosides administration, their metabolites appeared in plasma with a few minutes delay as compared to aglycones, which suggested that aglycones were absorbed already in the rat stomach. This observation was confirmed when absorption site was restricted to the stomach and absorption was shown to be independent of the vehicle pH used for administration.	[54]

№	Analyte/composition	Sample/matrix	Separation method	Detection method	Remarks	Ref.
38	Daidzein, Acetyldaidzin, Malonyldaidzin, Genistein, Acetylgenistin, Malonylgenistin, Glycitein, Acetylglycitin, Malonylglycitin Equol, *O*-Desmethylangolensin, Formononetin, Biochain A, Coumesterol	Soy foods, human urine, plasma, breast milk	HPLC gradient method	UV, fluorimetric detection, electrochemical detection, GC–MS, LOD: 0.53–3.28 pM	Acid, or enzymatic hydrolysis and solid phase, or solvent extraction were used for samples preparation.	[55]
39	Genistein, Daidzein, Equol, *O*-Desmethylangolensin	Human plasma, urine and feces	Isotope dilution capillary GC after purification by ion exchange chromatography, enzymatic hydrolysis and extraction with ethyl ether	MS	The authors concluded that GE is the most important isoflavone in plasma of the subjects that consumed the soy products, because it reached the highest concentration and had the longest half-life.	[56]
40	Genistein, Biochanin A, Daidzein, Formononetin, Coumestrol, Equol	Mice plasma and urine	C_8 HPLC after liquid–liquid extraction with ethyl ether column: NovaPak-C_8 (150 mm × 3.9 mm, 4 μm) flow rate: 1 mL min^{-1} mobile phase: acetonitrile – 0.05M ammonium formate buffer, pH 4.0 (27:73 *v*/*v*); isocratic method	UV and MS, LOD: 0.02 μg mL^{-1}	A specific, sensitive and facile assay employing only 50 μL of plasma or urine was developed. It permits replicate determinations of specimens obtained from a single mouse. The method is simple and easy to perform and it is quoted in many subsequent papers. In the work TSP-MS detection was employed to confirm the identity of the GE peak. Concentration estimated from the TSP-MS response (0.14 μg mL^{-1}) was in excellent agreement with the value determined by UV.	[57]

№	Analyte/com-position	Sample/ matrix	Separation method	Detection method	Remarks	Ref.
41	Isoflavones	Soy foods: soy bean seeds, soy bean hulls, soy flour, tofu, soy bean black, green bean, red bean, pin-to bean, peas, kala chana seeds and others, human urine	HPLC; column: NovaPak-C_{18} (150 mm × 3.9 mm, 4 μm); flow rate: 0.8 mL min^{-1} mobile phase: A: acetonitrile, B: acetic acid–water (10:90; *v*/*v*); gradient method	PDA, Fluorescence detector, LOD: 5–623 nM	Precise extraction and hydrolysis procedures described, resulting in reliable and reproducible quantification method.	[58]
42	Daidzin, Rhoifolin	Human plasma	HPLC; column: Intersil ODS-2 (250 mm × 4.6 mm, 5 μm); flow rate: 0.7 mL min^{-1} mobile phase: methanol–0.1 M ammonium acetate solution (pH 7.0) 33:67; isocratic method	UV (250 nm), LOD: 3.0 ng mL^{-1}	Sample preparation described; accuracy and sensitivity of the method determined.	[59]

Table II

Determination of isoflavones in plants and food samples

№	Analyte/ composition	Sample/ matrix	Separation method	Detection method	Remarks	Ref.
1	Genistein and Daidzein	Powdered milks and infant formulas	GC after ultra-centrifugation of dissolved formula solutions, SPE on C_{18} of supernatant and trimethylsilylation	MS, LOQ: 1 ng g^{-1} for B, 10 ng g^{-1} for D and G	High concentration of DA and GE (2050 and 6510 ng g^{-1} respectively) were detected in a soy-based powdered infant formula. The daily exposure of infants to DA and GE may be considered a risk factor. The authors advocate monitoring infant formula production and publicizing food safety concerns.	[60]
2	Isoflavone metabolites	Serum of mice fed soy proteins	HPLC	ESI-MS–MS, LOD: 0.018 mμM HGE 0.035 mμM DHD, LOQ: 0.04 mμM G, 0.08 mμM DHD	The study provides evidence for the presence of DHD in serum of mice fed diets containing soy proteins.	[61]
3	6″-*O*-Malonyl-daidzin, 6″-*O*-Malonylgenistin	Soybeans	HPLC		Comparison of isoflavones assay results vs. soybean storage time.	[62]
4	Daidzein, Genistein	Soy samples	HPLC	DAD MS	Time depended content and distribution of isoflavones in natural conditions of various parts of soy plants were determined.	[63]
5	Daidzin, Genistin, Glycitin, Daidzein, Glycitein, Genistein	Soy based infant food	LC	UV 262 nm and 250 nm, LOD: 0.02 – 0.05 μg g^{-1} formula, LOQ: 0.08–0.18 μg g^{-1} formula	Method of isolation of analytical samples by extraction and alkaline hydrolysis and its validation were described.	[64]
6	Genistein Daidzein	Soy samples	HPLC		Validation of HPLC method presented.	[65]

№	Analyte/ composition	Sample/ matrix	Separation method	Detection method	Remarks	Ref.
7	Genistein Daidzein	Soy-based infant foods	Gradient HPLC; Column C_{18} (300 mm × 3.9 mm with precolumn). Mobile phase: A: 10% acetic acid, B: ACN. Flow rate: 0.8 mL min^{-1}; v = 20 μL. Preparation sam-ples: extraction and hydrolysis from food	UV–visible; detection at 260 nm, LOD: 0.1 μg g^{-1} for solid samples; 0.05 μg mL^{-1} for liquid samples	Extraction and hydrolysis from food samples are described.	[66]
8	Puerarin Daidzin Genistin Daidzein Genistein	Kudzu root	Gradient HPLC. Column C_{18} (250 mm × 4.6 mm; 5 μm with guard column the sa-me packing). Mobile phase: A: acetic acid–water (pH 3.0), B: ACN, Flow rate: 1.0 mL min^{-1}; v = 30 μL	UV–visible Detection at 260 nm Combined with CIMS	Extraction and hydrolysis of isoflavones from food samples, especially from Kudzu root is discussed as well as derivatization and statistical analyses of results obtained by different procedures.	[67]
9	Phytoestrogens: Genistein, Daidzein, Equol, Formononetin, Biochanin A	Bovine milk	HPLC	ESI-MS–MS	Analysis of various milk samples was carried out and the occurrence of isoflavones was demonstrated in the concentration range 1–30 ng mL^{-1}.	[68]
10	Daidzein, Genistein	Soy foods	HPLC; column: Zorbax eclipse XDB-C_8 (150 mm × 6.0 mm, 5 μm); flow rate: 1.5 mL min^{-1}; mobile phase: A: water, B: methanol; gradient method	UV-DAD, LOD: 0.41–0.48 ng mL^{-1}	Automated on-line in-tube solid phase microextraction procedure was developed.	[69]
11	Isoflavones and isoflavone conjugates: 7-*O*-D-glucopyranosides and their respective 6″-*O*-malonates and acetates	Soy samples	Gradient HPLC. Column C_{18} (150 mm ×3 mm; 3 μm + 10 mm × 3 mm; 5 μm); Tem-perature column and detector 37°C; Sam-ples stored in autosampler at 10°C; Mobile phase: A: 50 mM sodium acetate buffer pH 5: MeOH (80:20 *v*/*v*), B: 50 mM sodium acetate buffer pH 5: MeOH:ACN (40:40:20 *v*/*v*). Flow rate: 0.3 mL min^{-1}; V = 10 μL	CEAD (coulometric electrode array detector) −1000 to +1000 mV, LOD: 38–93.3 fmol, LOQ: 3 times LOD	Sample pretreatment of soy products was applied. Statistical analysis of the results was provided.	[70]

№	Analyte/ composition	Sample/ matrix	Separation method	Detection method	Remarks	Ref.
12	Daidzein, genistein	Cereal and soy flours commonly eaten in Europe			Extraction, hydrolysis and assay comparison with use of synthetic standards.	[71]
13	Daidzin, Genistin, Glycitin, Daidzein, Genistein, Quercetin-diglycoside, Quercetin monoglycoside, Isorhamnetin glucoside	Peanut meal	HPLC; column: Zorbax SBC_8 (250 mm × 4.6 mm) flow rate: 1 mL min^{-1} mobile phase: A: acetonitrile B: water; gradient method	ESI, FABMS	Samples preparation and extraction procedures described, mass spectra interpreted.	[72]
14	Daidzein, Genistein, Formononetin, Biochanin A	Red clover samples	HPLC; column: Hypersil-BDS-C_{18} (250 mm × 4.0 mm 5 μm) flow rate: 1.0 mL min^{-1} mobile phase: A: 0.1 % sulfuric acid in water (pH 2.7), B: acetonitrile; gradient method	UV-PDA, LOD: 1.6–3.7 ng, LOQ: 2.0–10.0 ng	Procedures for hydrolysis and extraction was elaborated and method validation described.	[73]
15	Daidzein, Genistein	Soy food products	GC	C-IRMS (combustion-isotope ratio mass spectrometry)	Isotope labeled metabolites from soy plants were used as standards for foodstuff analysis.	[74]
16	Isoflavones and isoflavone conjugates: 7-*O*-D-glucopyranosides and their respective 6″-*O*- malonates and acetates	Soy foods: soy milk, soy flour, tofu, tempeh	HPLC	UV	Different solvents were tested for isoflavones extraction from soy foods. The best results were obtained with acetonitrile.	[75]

№	Analyte/ composition	Sample/ matrix	Separation method	Detection method	Remarks	Ref.
17	Genistein, Daidzein, Formononetin, Coumestrol, Biochanin A	Soybeans	HPLC; column: Phenyl Nova-Pack (150 mm × 3.9 mm 4 μm flow rate 1 mL min^{-1} mobile phase: acetonitrile – water (33:67 *v*/*v*); isocratic method	UV-PDA, APCI MS–MS, LOD: 47–224 nM	Extraction and hydrolysis procedures are described and discussed.	[76]
18	Genistein, Daidzein	Tofu Soy milk	HPLC	UV–visible Electrochemical MS thermospray		[77]
19	Daidzein, Genistein	Berry samples		ID-GC–MS-SIM. Isotope dilution gas chromatography–mass spectrometry in selected ion monitoring	Time dependent concentration of isoflavones determined in blood and urine after strawberry-meal in human subjects.	[78]
20	Isoflavones and isoflavone conjugates: 7-*O*-D-glucopyranosides and their respective 6″-*O*-malonates and acetates	Soybean	HPLC Column C_{18}		Comparison of isoflavones assay in various genotypes of soybean.	[79]
21	Daidzein, Genistein	Samples of food: apple, soybean, kudzu root, sunflower seed		ID-GC–MS-SIM. Isotope dilution gas chromatography–mass spectrometry in selected ion monitoring	Phytoestrogen content of foods and beverages of western, oriental, and Mediterranean diets.	[10]
22	Soybeans proteins	Soy foods	HPLC	DAD	Samples preparation described; statistical analysis performed.	[80]

№	Analyte/ composition	Sample/ matrix	Separation method	Detection method	Remarks	Ref.
23	Isoflavones and isoflavone conjugates: 7-*O*-D-glucopyranosides and their respective 6″-*O*-malonates and acetates	Soyfoods: Soymilk Tofu Soy meat Soy hamburgers	HPLC Column C_{18}, conditions of determination		Extraction and hydrolysis of food samples and statistical analysis of results are presented.	[81]
24	Daidzein, Daidzin, Formononetin, Isoformonomentin, Genistein, Genistin, Biochanin A, Prunetin	Seeds of pea (*Pisum sativum*) and mung bean (*Vigna radiata*)	HPLC, column: Nucleosil 100–5 C_{18} RP flow rate: 1.0 mL min^{-1} mobile phase: methanol – water; gradient method TLC, Ion-exchange TLC	RIA, UV 254 nm, MS, LOD: pg/tube	Samples preparation, extraction procedures and hydrolysis procedures are described.	[82]
25	Genistein-7-*O*-glucoside, Genistein-4-*O*-glucoside, 2′-Hydroxygenistein-7-*O*-glucoside, Apigenin-8-*C*-glucoside	Shoot of lupin (*Lupinus luteus* L)	GC	LSI, GC–MS, EI-MS	Extraction, column chromatography and preparative TLC were applied for samples preparation; structural studies were carried out.	[83]
26	Genistein	Plant extracts	Overpressured Layer Chromatography OPLC	Fluorescence	Samples preparation by hydrolysis and extraction are described.	[84]
27	Genistein, Daidzein, Biochanin A, Formononetin, Equol	Infant food	HPLC		Comparison of isoflavones exposure in infant diet and human food is discussed.	[85]

№	Analyte/ composition	Sample/ matrix	Separation method	Detection method	Remarks	Ref.
28	Isoflavones and isoflavone conjugates: 7-*O*-D-glucopyranosides and their respective, 6″-*O*-malonates and acetates	Soy foods: roa-sted soy beans, tofu, fried tofu, soyflour, tempeh, miso, soy milk, soy hot-dog, soy germ	Gradient C_{18} HPLC after extraction, evaporation and reconstitution, column: ODS-AM-303 (250 mm × 4.6 mm, 5 μm) flow rate: 1.0 mL min^{-1} mobile phase: A: 0.1% acetic acid in water, B: 0.1% acetic acid in acetonitrile; gradient method	PDA	Isoflavone concentration in soyfoods and soybeans can vary and are de-pendent on the soybean and process-sing conditions used to produce a particular food product. Authors determined precision of their me-thod, which is considered suitable for quality control of soy foods.	[86]
29	Isoflavones and isoflavone conjugates: 7-*O*-D-glucopyranosides and their respective 6″-*O*-malonates and acetates, Equol, *O*-Desmethylangolensin, Coumesterol	Soy foods, human urine, plasma, breast milk	HPLC gradient method	UV, fluorimetric detection, electrochemical detection, GC–MS, LOD: 0.53–3.28 pM	Acidic or enzymatic hydrolysis and solid phase, or solvent extraction were used for samples preparation.	[87]
30	Genistein, Biochanin A, Daidzein, Formononetin, Coumestrol, Equol	Soy foods: (beans, oils, flours, tofu); other beans and peas, human urine	HPLC, column: NovaPak-C_{18} (150 mm × 3.9 mm, 4 μm), flow rate: 0.8 mL min^{-1} mobile phase: A: acetonitrile, B: acetic acid–water (10:90; *v*/*v*); gradient method	PDA, Fluorescence detector, LOD: 5–623 nM	Extraction and hydrolysis procedures described, leading to fast and reproducible method of isoflavone aglycones determination.	[88]

№	Analyte/ composition	Sample/ matrix	Separation method	Detection method	Remarks	Ref.
31	Isoflavones and isoflavone conjugates: 7-*O*-D-glucopyranosides and their respective 6″-*O*- malonates and acetates	Soy beans and soy foods (milk, flour, proteins)	HPLC, column: Aquapore C_8 (250 mm × 4.6 mm) flow rate 1.5 mL min^{-1} mobile phase: acetonitrile – water–TFA, gradient method	UV at 262 nm API-MS	Extraction conditions are described and analysis of mass spectra is provided.	[89]
32	Isoflavones and isoflavone conjugates: 7-*O*-D-glucopyranosides and their respective 6″-*O*- malonates and acetates	Soybeans and soy foods	HPLC; column: YMC-pack ODS-AM (250 mm × 4.6 mm, 5 μm) flow rate: 1 mL min^{-1} mobile phase: acetonitrile – 0.1% acetic acid aq, gradient method	PDA, LOD: 100–185 ng mL^{-1}	Extraction conditions and hydrolysis procedures are described. Statistical analysis performed.	[90]
33	Genistein, Daidzein, and their glycoside conjugates	Soybean foods	HPLC, column: Brownlee Aquapore C_8 RP (350 mm × 4.6 mm), flow rate: 1.5 mL min^{-1}; mobile phase: acetonitrile – 0.1% TFA aq, gradient method	UV, MS	Samples preparation and extraction procedures described; variety of soybean from different sources were analyzed.	[91]
34	Daidzin, Genistin, Genistein, Daidzein, Coumesterol	Soy flakes	HPLC; column: Ultrasphere octadecylsilane (250 mm × 4.6 mm) mobile phase: A: methanol B: water; nonlinear gradient method	UV	Various solvents and solvent mixtures were tested for efficiency of extraction.	[92]

isoflavone content. This includes soy as a genetically modified organism and hundreds of its cultivars, selected for oil-quality and content and for amino acid composition. Isoflavone profile and content constitute another important genetic trait useful for chemotaxonomical classification, albeit not yet fully exploited [1–3]. Quantification of isoflavones in soy flour is an important task, not only for evaluation of cardiovascular risk reduction in the general population, but mainly because the flour is used in the manufacture of infant formula, resulting in exposure of babies fed with soy formula to gargantuan and possibly harmful cumulative doses [67]. In general, food analysis needs reliable quantification methods for isoflavones, compatible with the enormous variety of sample characteristics and content levels. The same applies, to an even greater extent, to functional food products (relatively new concepts accompanied by "enhanced function" and/or "disease risk-reduction" claims) [2,28].

Specification of herbal materials and of preparations derived from them constitutes another field, immediately related to human health, in which much improvement is needed [9–11,21–24]. Pharmacognosy textbooks and phytochemical data bases contain only elementary information about the main secondary metabolites of plants and seldom indicate how quantitative data were obtained. On the other hand, analytical procedures for separation of flavonoids quoted in chromatographic (HPLC) manuals and catalogs do not usually describe the principal characteristics of described method, which go beyond column type and detector settings. There is great public interest in dietary supplements, phytopharmaceuticals, and nutraceuticals, because of the growing cost of treatment with ethical (or generic) drugs, in which there has also recently been a serious crisis of confidence – one example is hormone-replacement therapy. But natural products-based pharmacology is, at least at present, no match for a pharmaceutical industry concerned with synthetic chemicals, mainly because the business of phyto-preparations is inadequately specified and poorly regulated in most countries, including the US, which is the main world market. Isoflavones are found in innumerable nutraceutical preparations. Their active ingredients are derived both from soy, and from other plants, for example red clover, black cohosh, alfalfa, or kudzu. Because their isoflavone constituents are estrogenic and their content may vary substantially, use of some preparations may raise legitimate health concerns, at least in some groups of patients (for example women with a high risk of hormone-dependent cancers) and their specification and quality is a serious matter. It has also been established, by independent academic authorities, that ma-

nufacturer declarations of the isoflavone content of marketed preparations are frequently substantially different from values obtained by analytical methods based on sophisticated separation–detection techniques [17,28,76].

Quantification of isoflavones is also of great importance in medical and clinical analysis. Because of sound epidemiological evidence of the beneficial effects of soy consumption (and hence isoflavone uptake) on human health, suitable biomarkers are needed, with reliable methods for their detection. Most accumulated data relating to soy food consumption in particular populations was, until recently, derived from voluntary questionnnaires, and must be verified by analytical measurements. Such tasks pose several serious technical problems, which require experiment planning as diligent as that usually applied to regular clinical trials, because of the genetic and metabolic polymorphism of any large cohort examined. It is currently assumed that the presence of isoflavone aglycones in urine and/or plasma can serve as a quantitative marker of soy consumption. Bioavailability of genistein and daidzein in man has been determined in several independent experiments and it has become clear that a relatively small fraction of a dose can be found in plasma as the unconjugated aglycone [17, 22,30]. These findings clearly demonstrate the need for simultaneous determination of a variety of metabolites and conjugates, which frequently differ dramatically in physicochemical properties and chromatographic retention indices [9,10,14,15]. The constantly evolving state of the art in clinical and phytochemical analyzes thus calls for periodic updates, focusing on the quality of the results achievable. The contents of Tables I and II result from critical sampling of recent work, mainly performed in connection with food and food supplement specifications, and trends in medicine, foreseeing application of plant isoflavones as a new generation of selective estrogen receptor modulators suitable for hormone replacement therapy, which is an important therapeutic task, corresponding to valuable segment of the pharmaceutical market.

CONCLUSIONS

Isoflavones are constituents of soy-rich diet and of numerous nutraceutical preparations. Their inherent estrogenic and antioxidant properties are believed to have beneficial effects on consumers' health. Recent trends toward regulation of, at least, some non-nutritional food constituents, for example phytoestrogens (which evoked an avalanche of publications over the last decade), are gradually being implemented, extending the harsh

quality requirements of medicines to agriculture and the food industry. The most advanced technical achievements in separation and detection techniques are currently setting standards for study and specification of isoflavones, starting from plant matrix, through food processing, to human intake, biodistribution, metabolism, and excretion [17–20]. Immediate gains from such developments include the realization that quite large inter-individual differences exist in isoflavone metabolism (e.g. equol production); this must influence future clinical study design.

Whereas 20 years ago the principal chromatographic methods for analysis of flavonoids were TLC and GC, in recent years HPLC has taken over almost completely. Separation of widely differing compounds (for example aglycones and their glucoconjugates) is a common problem in both phytochemical and clinical studies; this is now routinely resolved by use of HPLC on reversed-phase columns with gradient elution. Solvent extraction and SPE procedures are frequently used in biological sample preparation before quantification. For plant material and food products quantitative analysis is easier because liquid extraction methods are sufficiently effective and there is practically no limit on sample size and amount of pre-concentration, which results in widespread application of diode-array UV detectors. Biological samples, on the other hand, require much more sensitive detection devices, and mass spectrometry is extensively used for this purpose, in combination with soft ionization techniques or tandem MS detectors. Capillary electrophoresis, which seems theoretically well suited for separation of polyphenols, which ionize readily in basic buffers, has seldom been used for analysis of isoflavones. Combined with electrochemical detection it could, in the near future, offer high throughput capability, at viable cost. From non-chromatographic methods of quantification, different variants of immunoassays are gaining popularity for their sensitivity and specificity in a single-component detection, although chemical derivatization to raise suitable antibody is unavoidable. All three types of detection – radioactive isotopes, ELISA, and time-resolved fluoroimmunoassay (TR-FIA) have been applied to isoflavone aglycones [14,17]. MALDI–TOF-MS has been shown to provide all the necessary structural information for rapid (ca. 100 samples h^{-1}) identification of isoflavones in soy samples, although quantitation with this technique is currently beyond reach. Most of the published procedures collected in Tables I and II offer nanomolar (nM L^{-1}) sensitivity. Femtomolar detection limits seem to be achievable, especially for fluorescence or electrochemical-array detectors. Navigating through a great abundance of analytical data concerning isoflavones shows

clearly that current requirements of pharmaceutical product quality control are seldom met by procedures published in scientific journals. Although acceptable linearity is usually demonstrated, limits of: detection and quantification (LOD and LOQ) are rarely determined and other method-validation data (intra and interbatch accuracy, selectivity, precision, and robustness) are not addressed (Tables I and II). Nevertheless, progress in the analysis of isoflavone-type compounds achieved in recent years can secure further method development suitable for conducting new product development or clinical trials of new drug candidates, under GLP or GCP conditions.

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