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Review

Current trends in isolation, separation, determination and identification of isoflavones: A review

Isoflavones are natural substances which elicit a number of physiological effects in living organisms. The substances are synthesized in plant tissues as protective agents against biotic stress (*i.e.* bacterial infection). Isoflavones are also an important dietary constituent in human nutrition. Modern trends in studies of isoflavones in plant materials and foodstuffs and procedures for chemical analyses of isoflavones in human body fluids and plant tissues are discussed in this review. Highly effective extraction and purification techniques, *i.e.* solid-phase extraction, accelerated-solvent extraction, and Soxhlet extraction, are presented. Latest procedures in chromatographic separation of isoflavones that apply different types of sorbents are described. Immunochemical analysis, electrochemical sensing of isoflavones, and spectrometric and other analytical techniques and their applications are also mentioned. Special attention is paid to the highly selective and sensitive technique of mass spectrometry and its application for identification of isoflavones and their glucosides in plants. Studies of interactions of isoflavones with cell receptors and a number of biologically active substances such as DNA and proteins are described. The review does not intend to give a complete overview of the topics considered but rather to present modern and most recent methods used in studies of isoflavones.

Keywords: Chromatography / Daidzein / Electroanalysis / ELISA / Estrogens / Extraction technique / Flavonoids / Food and agricultural research / Genistein / Glycitein / Human health / Isoflavones / Isolation / Mass spectrometry / Phytoestrogens

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1 Introduction

Isoflavones belong (together with flavanones, flavonols, catechins, anthocyanidins, and others) to a widespread group of natural products called flavonoids. They are secondary plant metabolites (polyphenols) eliciting a huge number of physiological responses in humans and other mammals. Isoflavones are well known as anti-carcinogenic substances and they have found several applications in the prevention of cardiovascular diseases [1, 2]. In addition to the beneficial effects of isoflavones on human health, several publications on their toxic effects that are connected with the applied dose and many other factors [3] have been described. The substances are called phytoestrogens since they exhibit estrogenic activity (similar effects to estradiol hormones). The effects are often used to treat symptoms of menopause, post-menopause osteoporosis, and other estrogen-related disorders. The chemical structures of three selected isoflavones (aglycones in their native form) are presented in Fig. 1 together with the structure of 17-β-estradiol.

The required intake of isoflavones by the human body can be fully met by consumption of food. Products based on legumes – soy (*Glycine max*), peas, alfalfa, or red clover, and other members of Fabaceae family – belong to the most frequently used products. In addition to isoflavones, some other flavonoids, such as lignans (see review

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Abbreviations: **APCI,** atmospheric pressure chemical ionization; **ASE,** accelerated-solvent extraction; **CPE,** carbon paste electrode; **DAD,** diode-array detection; **ED,** electrochemical detector; **FIA,** flow injection analysis; **GCE,** glassy carbon electrode; **HSCCC,** high-speed counter-current chromatography; **MAE,** microwave-assisted extraction; **RR,** rapid resolution; **SFE,** supercritical-fluid extraction; **SPI,** soy protein isolates; **TR-FIA,** time-resolved fluorescence immunoanalysis; **UP,** ultra-performance; **UV-Vis,** ultraviolet-visible

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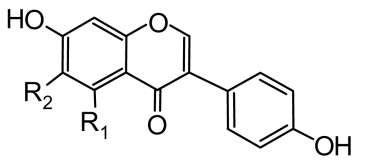


R_1	R_2	aglycones
H	H	daidzein
OH	H	genistein
H	OCH_3	glycitein

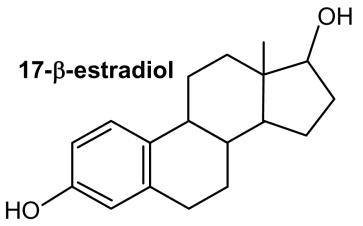
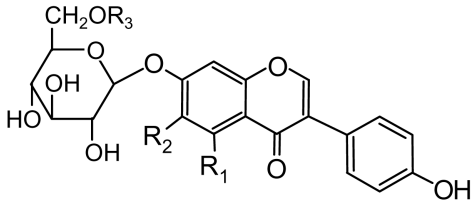


Figure 1. Chemical structures of isoflavone aglycones and 17-β-estradiol.



R_1	R_2	R_3	glucoside
H	H	H	daidzin
OH	H	H	genistin
H	OCH_3	H	glycitin
			acetylglucoside
H	H	$COCH_3$	acetyldaidzin
OH	H	$COCH_3$	acetylgenistin
H	OCH_3	$COCH_3$	acetylglycitin
			malonylglucoside
H	H	$COCH_2COOH$	malonyldaidzin
OH	H	$COCH_2COOH$	malonylgenistin
H	OCH_3	$COCH_2COOH$	malonylglycitin

Figure 2. Chemical structures of isoflavone glucosides (presented in Fig. 1) and their acetyl- and malonyl-derivatives.

[4]), are often presented as members of the group of phytoestrogens. Plants synthesize isoflavones, and of course numerous other groups of polyphenolic substances, as protective substances when bacterial and/or other infectious agents attack plant organs. They also participate in many regulation mechanisms in plant cells. Isoflavones are present mainly in the form of β-glucosides and their acetyl- or malonyl-derivatives (see Fig. 2) in plant tissues.

Due to their biomedical importance and wide applicability, studies of isoflavones and development of a new analytical instrumentation for their unequivocal identification in plants are of great interest to many research teams worldwide. The fact that about 500 papers dealing with isoflavones and more than 1700 papers contributing to the chemistry of flavonoids appeared in 2006 (source: ISI Web of Science) confirms the enormous interest in this group of metabolites.

At present, extraordinary progress in isolation, purification, and separation techniques and in detection instrumentation is promoting their application in the analytical chemistry of phenolic substances, not only in legumes but also in other groups of plant families that are rich in isoflavones. Similar interest is also being directed to less common plants with lower concentrations of isoflavones, such as some medicinal plants, spices, *etc*. Several recent review papers deal with the problems of exact determination and identification of flavonoids (or phytoestrogens) and/or especially with isoflavones [5 – 8].

Problems of isolation and purification of crude extracts of isoflavones using modern extraction techniques and their subsequent separation, determination, and identification in plant materials and food products by selected chromatographic and electromigration methods (*i.e.* HPLC, CE, *etc*.) are mainly described in the present review. The most recent developments in detection of isoflavones and their exact identification in plants by UV-Vis spectrophotometry, mass spectrometry, immuno-analytical methods, electrochemical detection (ED), and other techniques are also discussed. The main aim of the review is to present modern and most recent methods in studies of isoflavones (separation techniques such as column and planar chromatography and gas chromatography are not included).

2 Occurrence of isoflavones in plants

The 3-phenyl-chromen-4-one skeleton bearing different substituents is the basic chemical structure of isoflavones. Aglycones like daidzein (7,4-dihydroxyisoflavone), genistein (5,7,4-trihydroxyisoflavone), and glycitein (7,4-dihydroxy-6-methoxyisoflavone) belong to the simplest structures (see Fig. 1). Aglycones are relatively scarce in plant tissues and they are present in low concentration. Their glucoside conjugates prevail in the tissues (see Fig. 2; for details see [9]). Conjugates of genistein and daidzein, *i.e.* genistin (genistein-7-*O*-β-D-glucoside), 6-*O*-

malonyl-genistin, daidzin (daidzein-7-*O*-β-D-glucoside), and 6-*O*-malonyl-daidzin and conjugates of glycitein, are the major isoflavones of soybeans. Red clover, which contains mainly formononetin (4-methylated form of daidzein) and biochanin A, is another important source of the isoflavones. The presence of the substances in plants is greatly influenced by cultivation and climatic conditions in which the plants are cultivated or planted.

Due to the enormous progress in detection techniques, numerous other isoflavones have so far been identified not only in legumes, but also even in non-leguminous taxa such as Convolvulaceae, Cyperaceae, Brassicaceae (*A. thaliana*), and many other families [10]. For example, identification of glaziovianin A in *Ateleia glazioviana* [11] belongs to the most recent discoveries. New isoflavones were also identified in rhizomes of *Belamcanda chinensis* [12] or new prenylisoflavone derivatives were discovered in roots of *Hedysarum scoparium* [13]. Isolation of new isoflavones also influences research in biomedicine and pharmacochemical sciences. A cytotoxic activity of glaziovianin A against human HL-60 leukemia cells was confirmed. More detailed information on the presence of isoflavones across plant materials and their application as systematic markers can be found in the literature [10, 14].

3 Stability of isoflavones

The stability of plant natural substances is limited by a huge number of physical (light, temperature, *etc*.) and chemical factors (pH, selection of a suitable solvent, *etc*.). This fact must be taken into account during any manipulation and preparation of a sample for analysis. In addition, information about the stability of isoflavones is very important in preparation and storage of meals and/or in stabilization of pharmaceutical preparations rich in isoflavones.

Isoflavone substances are sensitive to irradiation (UV photodegradation). Solutions of daidzein and formononetin in various organic solvents (acetonitrile, methanol, ethanol, hexanol) were found to undergo photodegradation on exposure to solar-type radiation [15]. Degradation of the isoflavones can be detected by a decrease of absorbance at their absorption maximum (around 250 nm) and changes in their fluorescence emission. It is therefore better to store standards, samples, and all preparations containing isoflavones in the dark.

It was confirmed that temperature also influences the stability of isoflavones. Mathias *et al*. [16] studied the stability of malonyl- and acetyl-conjugates of daidzin and genistin at 25, 80, and 100°C and at different pH values. The tested β-glucosides and their derivatives undergo degradation (hydrolysis) mainly at elevated pH values (mainly at pH values above 10 – alkaline hydrolysis) and the degradation is negligible at room temperature 25°C. The stability of isoflavones was studied in extracts of red clover *Trifolium pratense* [17]. The authors focused their interest on the analysis of formononetin-β-glucoside malonate and biochanin A-β-glucoside malonate (the major isoflavones present in red clover) using RP-HPLC techniques directly coupled to a mass spectrometric, UV-Vis, and fluorescence detectors. At elevated temperature (83°C), hydrolysis of both derivatives takes place, forming β-glucosides and free aglycones. The temperature of storage primarily influences the stability of derivatives of isoflavones in extracts. Since the aglycones are formed during hydrolytic processes, degradation can be reduced or eliminated by freezing the samples.

Temperature is also an important factor in the treatment of foodstuffs containing isoflavones. For example, degradation of genistein [18] and slow decrease of its concentration was observed during heating of soymilk. Detail information about both alkaline and acid hydrolysis can be found in reference [19]. Controlled hydrolysis of β-glucosides and their acetyl- and malonyl-derivatives can be used for quantification of isoflavones in real samples. Conjugated forms of isoflavones can be transformed into their aglycones using hydrolytic processes and these can be easily quantified. In addition, commercial standards and certified reference materials for determination of isoflavones are produced in the form of aglycones in most cases. The presented results are very important for sample preparation and for analyses of isoflavones and technology of food products and pharmaceutical preparations.

4 Analysis of isoflavones

Analysis of isoflavones can be divided into several consecutive steps: (i) sample collection; (ii) disintegration of plant tissues and homogenization of a sample; (iii) extraction and purification of crude extracts; (iv) separation and identification of individual substances; (v) detection and quantification; and (vi) interpretation of obtained results. Each individual step needs special care; in particular, possible interactions of isoflavones with individual constituents of a matrix and their ability of their conjugates to undergo hydrolysis must be taken into account (see Section 3). Modern techniques of isolation, separation, and detection of isoflavones are described in the following sections.

4.1 Extraction methods for isoflavones

Extraction of target analytes from complex real samples is the most important step on the way from the raw sample to quantitative analytical determination. The aim of extraction is to remove all target analytes from the sam-

ple, usually as a small amount of liquid suitable for the appropriate analytical method. A good extraction method should at least be fast: it may take tens of minutes, but not many hours. As for the extraction selectivity, co-extracted compounds should neither interfere with the determination of the analytes of interest nor cause any trouble during the determination (peak overlap, masking, damage to chromatographic column, *etc.*). Good recovery is crucial for a practicable extraction method.

One hundred years ago, solvent extraction was a common method (Soxhlet extraction has been known since 1879), and newer extraction methods subsequently came into use as they were invented. Nowadays, liquid extraction with a two-phase solvent system is used for extraction from plant materials [20]; and for preparative isolation a solvent reflux system with 80% aqueous ethanol has also been reported, as well as liquid-liquid extraction using a mixed solvent of 10% *n*-butanol and 90% ethyl acetate [21], giving a recovery close to 70%. Even lower recoveries are still accepted from some types of matrices, provided they are coupled with good reproducibility, *e.g.* about 60% from rat tissue [22].

Ultrasound-assisted methods substantially shorten the time needed for liquid extraction, *i.e.* from 6 hours to 20 minutes in the case of garden sage [23]. High intensity ultrasound-assisted extraction (sonication) has been used for extraction of isoflavones and oils from soybeans, using hexane, 2-propanol, and a 3:2 hexane – isopropanol mixture [24]. Best yields (62.3%) were achieved after extraction for 3 hours. Isoflavone derivatives (daidzin, glycitin, genistin, and malonylgenistin) can be extracted almost quantitatively (80 – 90%) from freeze-dried ground soybeans in 20 min with 50% ethanol at 60°C using ultrasound-assisted extraction [25]. Longer extraction times, however, lower the recovery. The method is used for routine analyses [26].

Extraction efficiencies exceeding 90% of daidzein, genistein, formononetin, and biochanin A were achieved via sorption on Speed ABN cartridges [27]. The method, which is generally used as a pre-clean step, was used for fast determination of isoflavones in plants, foods, and other biological materials. The method was further developed into two-dimensional solid-phase extraction (2-D SPE) that allowed simultaneous extraction of fourteen isoflavone glucoside malonates and six acetylglucosides from red clover [28]. Specific cases of co-elution of two or more compounds causing interference during the determination of secondary metabolites in plant materials were further investigated and solved [29].

Seven basic isoflavones were extracted quantitatively from various "soy meat" products [30] by the means of accelerated solvent extraction (ASE). According to the comparative study presented in the paper, each extraction technique favored a different group of compounds. ASE combined with sonication and SPE clean-up was applied for the extraction and isolation of isoflavone aglycones and glucosides from soy plants parts, with recoveries of 96 – 106% [31].

Microwave assisted extraction (MAE) appears to be a fast and reliable method for soy isoflavones [32]. Recovery of 97 – 103% of malonyldaidzin, malonylglycitin, malonylgenistin, acetyldaidzin, acetylglycitin, acetylgenistin, daidzin, glycitin, genistin, daidzein, glycitein, and genistein was achieved at 50°C in 20 min with 50% ethanol as extracting solvent. In the case of runner peanuts, a comparative study proved [33] that microwave-assisted and Soxtec methods were better for isoflavone content quantification, while sonication or stirring was the method of choice for determination of isoflavone composition (aglycones and glucoside conjugates).

Supercritical fluid extraction (SFE) is used both for analytical purposes and for the preparation of functional foods [34], such as soy protein isolates (SPI). From soy hypocotyls, isolates containing 640 mg of isoflavones/100 g of SPI were prepared at 35 MPa, and 45°C during 120 min, and 83.7% of the isoflavone content was glycitin, daidzin, and genistin. SFE with continuous on-line modifier addition to the extraction phase was applied [35] to the extraction of daidzin, glycitin, genistein, ononin, daidzein, glycitein, sissotrin, formononetin, and biochanin A from red clover (chromatograms of real samples after SFE are shown in Fig. 3). According to a comparative study of daidzin and genistein recovery, SFE was slightly more efficient than pressurized solvent extraction and much better than IKA Soxhlet or sonication. Although the yields were extraordinarily high, good selectivity seems to be the most valued factor in the paper, especially avoiding the co-extraction of chlorophylls.

Two basic approaches prevail: intensive combination of several methods, leading to both quantitative recovery and high selectivity in a small extract volume, or selection of a less quantitative, but reproducible, easy-to-perform, and automatable method. In the first case, coupling of sonication, a method good in removing analyte from the matrix, and SPE seems to be the best choice. Short ultrasound-assisted treatment affects the matrix positively and makes further transport of analyte from the matrix to the extraction solvent much easier. After sonication, a strong and effective solvent should be used to free the analytes from the sample matrix, especially from active centers – a method such as SFE, MAE, ASE, or enhanced IKA Soxhlet. Crude extract is processed by SPE, where prospective harmful compounds are kept in the cartridge together with those causing co-elution with target analytes, and the extract is pre-concentrated to an acceptable volume (*ca.* 3 mL) at the same time. This approach is quite intensive and may be costly, but gives the required recovery, selectivity, and precision. Much

more concentrated and cleaner extracts allow the determination of these analytes in plant samples containing very low concentrations of isoflavones, and the investigation of larger groups of analytes. The improvements in isoflavone extraction methods accomplished in the last decade are summarized in de Rijke's review [5].

4.2 Liquid chromatographic methods

Grynkiewicz *et al*. [36] recently described the separation of isoflavones using chromatographic techniques. Much detailed information about chromatographic separation of flavonoids can be found in papers [5 – 8].

Thin-layer chromatography and polyamide chromatography prevailed over the other techniques for separation of isoflavones in the past. Classical liquid chromatography techniques and several modifications thereof using modern instrumentation are currently the most frequently applied techniques. Separation on reversed phase sorbents C_{18} (possibly C_8) is one of the most preferred. Separation of isoflavones on reversed phase columns is based on hydrophobic interactions of individual isoflavones with the stationary phase. Retention times of separated substances depend primarily on their solubility in water. It was confirmed that retention time on RP columns increases with increasing hydrophobicity of the analytes. Generally, the content of organic solvent in a mobile phase can greatly influence retention times. The hydrophobicity of individual isoflavones increases in the order β-glucosides, malonyl-, and acetyl-β-glucosides, and free aglycones are the most hydrophobic (for details see [37, 38]). Several other factors influence the retention times of separated isoflavones, *i.e.* their affinity to the stationary phase, which can be modified with different functional groups, composition of the mobile phase, application of gradient elution, column temperature, *etc*. HPLC separation of flavonoids in foodstuffs and food products on reversed phases is reviewed in [39].

In most cases aglycones and glucosides are separated together with their derivatives in a single run on an RP column. Isocratic elution is usually insufficient and a suitable gradient therefore has to be optimized for gradient elution. It usually starts at *ca.* 10% v/v of organic modifier (acetonitrile or methanol are the most suitable modifiers, for details see [39]). Separation of glucosides improves with gradually increasing modifier content. Aglycones are eluted from the stationary phase only at higher contents of organic modifier. A mixture of daidzin, glycitin, genistin, ononin, daidzein, glycitein, sissotrin, genistein, formononetin, and biochanin A was completely separated in less then 6 min [40] using optimized linear gradient elution with 0.2% formic acid and acetonitrile. Later [35], complete separation was accomplished in even less then 4 minutes by elution with a mobile phase consisting of 0.3% acetic acid and acetonitrile (Fig. 3).

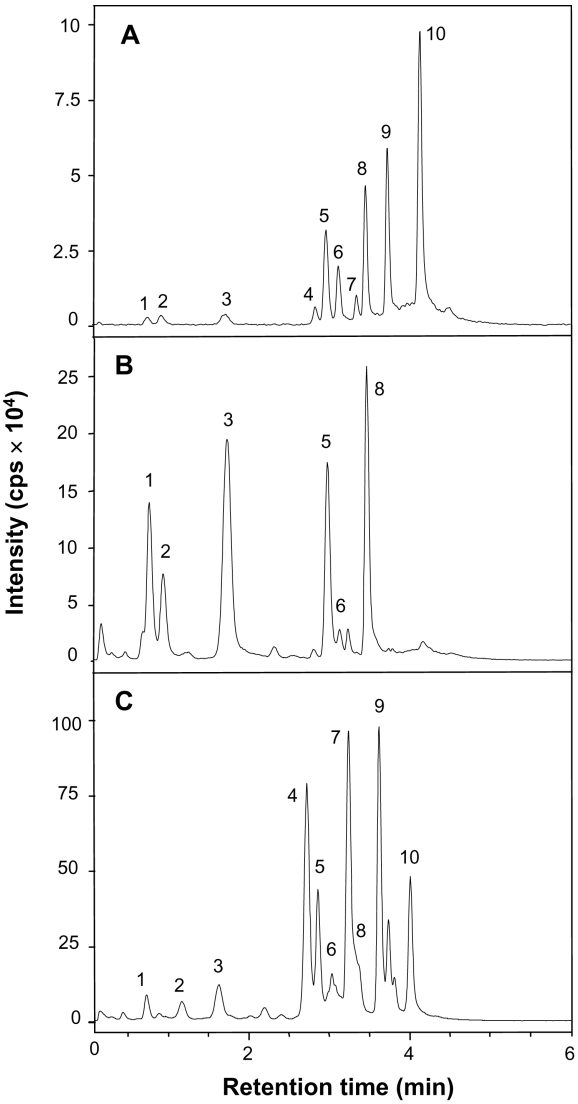


Figure 3. HPLC/MS chromatograms of (A) isoflavones and real samples after supercritical fluid extraction: (B) soya bits, (C) red clover. HPLC column: Atlantis dC_{18} (20 × 2.1 mm, 3 μm), mobile phase: 0.3% v/v acetic acid and acetonitrile (solvent B), linear gradient elution (v,%): from 13 up to 20% B from start to 1.25 min, up to 30% B to 1.61 min, up to 35% B to 2.0 min, up to 40% B to 2.25 min, up to 50% B to 2.57 min, and followed by a negative gradient to 13% B to 3.86 min, flow rate: 0.68 mL/min, column temperature: 36°C. For other details see [35]. Peak identification: 1 – daidzin, 2 – glycitin, 3 – genistin, 4 – ononin, 5 –daidzein, 6 – glycitein, 7 – sissotrin, 8 – genistein, 9 – formononetin, 10 – biochanin A.

Application of columns of smaller inner diameter and mainly with a smaller stationary phase particle size and application of new technologies in chromatographic instrumentation are preferred in chromatographic sep-

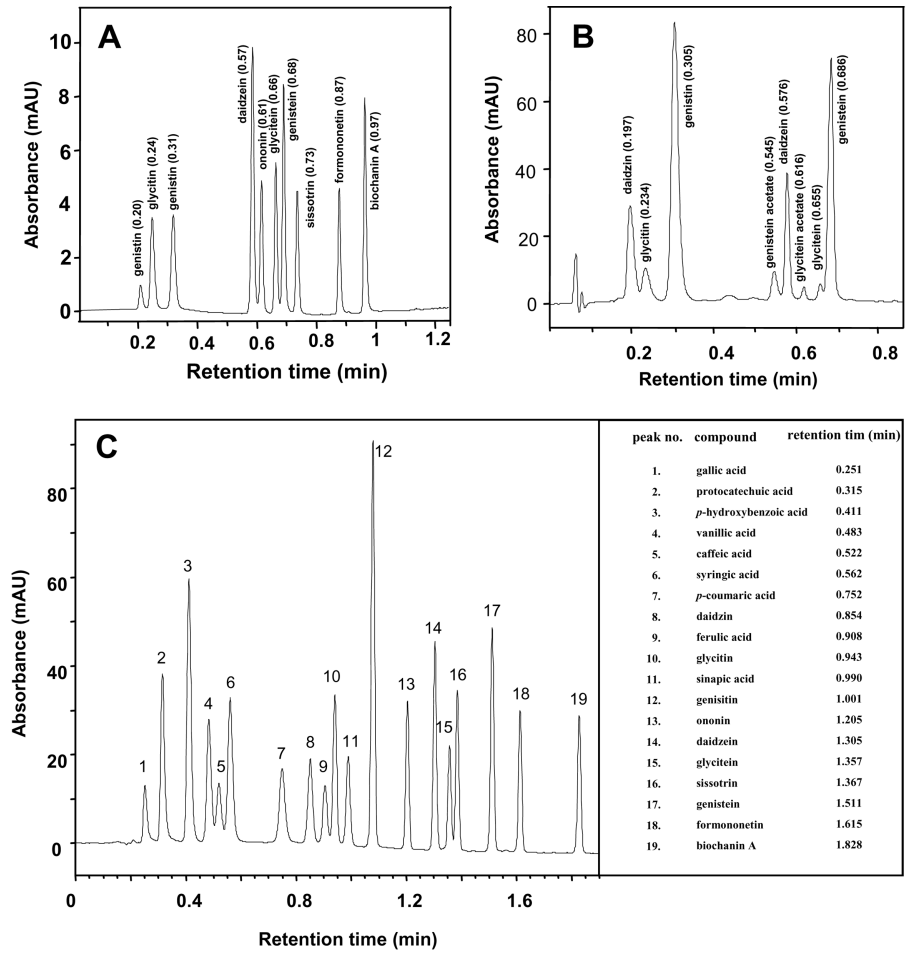


Figure 4. HPLC/UV-Vis chromatograms of isoflavones. Application of rapid-resolution (A, B) and ultra-performance (C) technology. Methanolic solutions of isoflavone standards (A) and in a case (B) real-sample (soy bits after modified Soxhlet extraction) were analyzed. Parameters of measurement (A, B): Zorbax SB C_{18} (30 × 2.1 mm, 1.8 μm), mobile phase: 0.2% (v/v) acetic acid and methanol (solvent B), linear gradient elution: 0 min 22% B, 1.0 min 80% B, 1.4 min 100% B, 1.8 min 22% B, flow rate 1.4 mL/min, column temperature: 80°C; for other details see ref. [41]. Parameters of measurement (C): Zorbax SB-CN (50 × 2.1 mm, 1.8 μm), mobile phase: 0.3% (v/v) acetic acid and methanol (solvent B), linear gradient elution (v,%): 0 min 10% B, 0.5 min 10% B, 1.8 min 45% B, 3 min 10% B, flow rate: 0.9 mL/min, column temperature: 58°C, injection volume: 1 μL; UV-Vis detection at 270 nm was used, concentration of analyzed compounds: 1 ng/mL.

aration of isoflavones at present*. Columns packed with RP sorbents of particle size below 2 μm are very suitable for separation of isoflavones. They allowed reduction of retention times to less than 60 s for complete separation of 10 isoflavones. The method was applied for analysis of real samples of soy food and plant materials from *Trifolium pratense*, *Iresine herbsti*, and *Ononis spinosa* in less then 2 min [41] (see Figs. 4A, B). Similarly, using a cyanopropyl stationary phase (particle size <2 μm), which is less hydrophobic then classical C_{18}, a mixture of phenolic acids and selected isoflavones was completely separated with retention times not exceeding 1.8 min for all substances (chromatogram shown in Fig. 4C). More hydrophilic columns such as C_{18} are more suitable for analyses of samples that need simultaneous separation of isoflavones and other phenolic substances of more polar character. The above-mentioned technology was also applied for separation of estrone, estradiol, estriol, ethynylestradiol, and the isoflavones: genistein, daidzein, and biochanin A (retention times 2.39, 2.05, and 3.25 min) using gradient elution with acetonitrile and water and quadrupole time-of-flight (QTOF) MS detection [42]. Indisputable advantages coming from the application of new technologies, including the coupling to MS detectors, are presented in paper [43].

Also columns with a monolithic stationary phase have found wide applicability in separation of isoflavones.

* The new high pressure technique using columns packed with sorbents with particle size under 2 μm is known as a rapid-resolution and/or ultra-performance LC (HPLC respectively) in the commercial sphere.

Monoliths belong to the separation media without interparticular space. The stationary phase of a monolithic column can be presented as a single separation particle of a polymeric material [44, 45]. Using a monolithic silica-based reversed-phase column, isoflavones in soy extracts were separated [24] with a mobile phase consisting of acetonitrile and acetic acid. A mobile phase consisting of acetic acid and methanol was applied for separation of daidzein, genistein, glycitein, and their glucosides and acetyl- and malonyl-derivatives. C_{18} monolithic stationary phase was used for quantification in soy food samples. Separation of 12 isoflavones was complete in *ca.* 10 min and very good resolution was obtained for all chromatographic peaks [26]. The separation of 11 aglycones of flavonoids in less then 15 min [46] is one of the latest applications of a monolithic reversed phase.

Complications connected with the separation of phenolic substances can be also solved using 2-D chromatography, which is based on connection of two different columns. Individual columns are suitable for separation of a selected group of substances present in a sample. The combined action of the two columns allows separation of mixtures that cannot be separated on a single chromatographic column or whose separation is not effective. A combination of a polyethylene glycol-silica column and C_{18} or zirconium-carbon column was used for separation of phenolic antioxidants [47].

Application of a non-porous stationary phase can be a valuable alternative in HPLC of flavonoid compounds. The column was used for separation of flavonoids in samples of *Filipendula ulmaria* [48]. The stationary phase covers the surface of non-porous micro particles. The thin film simplifies mass transport in the stationary phase. This stationary phase is not used for analyses of flavonoid substances on a larger scale.

High-speed counter-current chromatography (HSCCC) was also recently applied for separation of flavonoid substances. The technique is based on liquid/liquid separation. It was used for separation of flavonoid substances in medicinal plants used in traditional Chinese medical practice [49]. HSCCC is often used for separation of specific groups of substances. The isolated fraction(s) is analyzed by classical HPLC. The HSCCC has recently found wide applicability in the separation of different phenolic substances.

4.3 Other separation techniques

In the previous decade an enormous increase was observed in the number of papers dealing with the application of electromigration methods to the separation of natural substances. Numerous electromigration methods were applied for studies of flavonoids and/or isoflavones (see review by Herrero *et al*. [59]). Their ease of coupling to sensitive electrochemical detectors (EDs) is the main advantages of electromigration techniques. This is due to the applicability of very simple background electrolyte systems (mainly borate buffer in the mM concentration range).

Application of electrochemical detectors in chromatographic techniques is limited due to presence of organic modifiers in mobile phases. In addition, application of some common buffers limits both techniques if ESI-MS detectors have to be used. In addition to the conventional capillary zone electrophoresis also other electromigration techniques, *i.e.* capillary electrochromatography and micellar electrokinetic capillary chromatography, and possibly also capillary isotachophoresis, are used.

CZE is the most commonly used electromigration technique. Procedures for a highly effective separation of isoflavones were first introduced in the 1990s [50]. Results obtained with CZE were compared with those obtained by HPLC separation [51]. Isoflavones were determined in soy, lupine, and pea using both separation techniques and UV-Vis-diode array detectors (DAD). CZE separation was much faster than HPLC. On the other hand the HPLC separation is more selective for identified isoflavones. This applies mainly to various real samples.

CZE in combination with EDs was also employed [52] for the analysis of puerarine, daidzein, and rutin in *Pueraria lobata,* a plant used in traditional Chinese medicine (for common cold, influenza, and other ailments). A carbon disc (300 μm diameter) as a working electrode was positioned in wall-jet configuration. A working potential of 0.9 V was selected as the best on the basis of hydrodynamic voltammograms. Sub-μM concentrations of isoflavones could be detected using the technique. The same electrochemical detector was used for MEKC analyses of puerarin and daidzein in plant materials and pharmacological preparation from Puerariae Radix (a mixture of disintegrated roots of *P. lobata* and *P. thomsonii*) [53]. Sodium dodecyl sulfate (SDS) was used as a surfactant for the pseudo-stationary phase. Samples were injected electrokinetically, applying 18 kV for 10 s.

Recently, MEKC has been widely used for separation of sparingly water-soluble polyphenolic compounds and similar substances. Biochanin A, formononetin, genistein, and daidzein can be determined in red clover [54] using MEKC in combination with a UV detector. SDS was used as a pseudo-stationary phase. It was shown that more effective separation of the substances is possible in the presence of an organic modifier (ethanol 5% v/v) in a mobile phase (30 mM borate buffer). The time for the MEKC separation of the above-mentioned isoflavones did not exceed 20 min in most cases. In contrast to CZE, MEKC also allows effective separation of hydrophobic aglycones that are sparingly soluble in the buffers used in CZE. The problem can be partly solved in the presence of a small amount of dimethyl sulfoxide and/or by using alkaline buffers [55].

Capillary electrochromatography is another method that can be used for separation of isoflavones. The technique is based on separation of analytes in a capillary filled with a sorbent. In contrast to HPLC, the separation is driven by an electric field. Very good results were obtained using CEC with a monolithic column. A lauryl acrylate stationary phase polymerized directly inside the capillary was used for separation of a mixture of daidzein, genistein, and glycitein and their conjugates in soy-based infant formulas [55].

In addition to UV and ED detectors, electromigration column separations can also be directly coupled to mass-selective detectors [56] and/or fluorescence detectors. Generally, electromigration techniques are used not only for identification of isoflavones in various plant materials but also for studies of chemism (*i.e.* determination of ionization constants [57], study of influence of UV-B irradiation on stability of isoflavones [58], *etc*.). The pK_a values of isoflavones can be determined from the dependence of electrophoretic mobility on the pH of the buffer. The pK_a values of genistein (9.5), daidzein (9.55), glycitein (9.73), and others were determined using McLeold and Shepherd's technique.

More details of electrophoretic techniques and their applications in the analyses of isoflavones (and also other flavonoid substances) can be found in recent papers by Herrero *et al*. [59] and Li *et al*. [60].

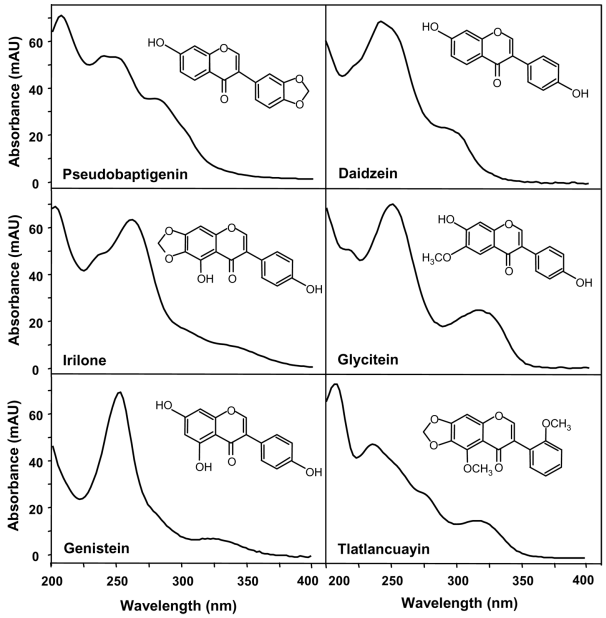


Figure 5. UV-Vis absorbance spectra of isoflavones in 0.2% (v/v) acetic acid and methanol. For other details see Fig. 4A and [41].

4.4 Identification of isoflavones and sensitive detection

4.4.1 Spectrometric methods

UV-Vis detectors, and mainly diode array detectors (DAD) that can operate in a wide range of wavelengths, are the detectors most often applied in chromatographic and/or electromigration separations [29]. The UV-Vis spectra of many isoflavones are very similar. Thus their applicability in identification of isoflavones is very limited since approximately 700 isoflavones are present in different plant materials. For illustration the UV-Vis spectra of pseudobaptigenin, daidzein, irilone, glycitein, genistein, and tlatlancuayin are presented in Fig. 5.

MS detectors that can detect individual aglycones of isoflavones, their derivatives or glucosides, and their other conjugates are the most effective tools for detailed studies of separated substances and for their identification. In addition to selective analyses, it is possible to apply mass spectrometry for structural analysis [61]. The combination of column separations with nuclear magnetic resonance (see review [62]) or MS and/or tandem MS [63] can be used in determining the structures of isoflavones.

Electrospray ionization, which can be connected to LC or CE, is the most common technique for ionization of a sample. Matrix-assisted laser desorption, atmospheric pressure chemical ionization (APCI), thermospray ionization [64], and others (see in [65]) belong to the other ionization techniques. Very good results were obtained using ESI-quadrupole MS in combination with liquid chromatography after SFE [35] with LODs for aglycones of 0.2 – 1.0 fmol and for glucosides of 1.3 – 3.6 fmol per injection. A fragmentation voltage 100 V was used and ESI was performed in negative mode. The same MS detector was earlier successfully used for determination of an isoflavone profile in *T. pratense*. It was also used for identification of their glucosides [28] or in detection of isoflavones in soybean food samples (LOD for daidzin/genistin were 1.2/1.6 fmol and 1 – 3 fmol for daidzein, genistein, formononetin, biochanin A, and ononin [30]). Each individual isoflavone is characterized not only by its molecular ion, but mainly by specific fragmentation products. Fragmentation is controlled by the so-called retro-Diels-Alder reaction, as shown in the case of daidzein in Fig. 6A. A study dealing with the fragmentation of isoflavones in negative electrospray ionization was presented by Kang *et al*. [66] and information about the fragmentation of glucosides were published in [9] (fragmentation of glucosidic conjugates of irilone is shown in Fig. 6B).

Coupling of LC with MS [67] numbers among the other modern techniques. Its application was described recently in a paper dealing with the determination of acetyl glucoside isoflavones and their metabolites in human urine. Also very selective are the newly developed techniques of MS/MS that were used for determination of

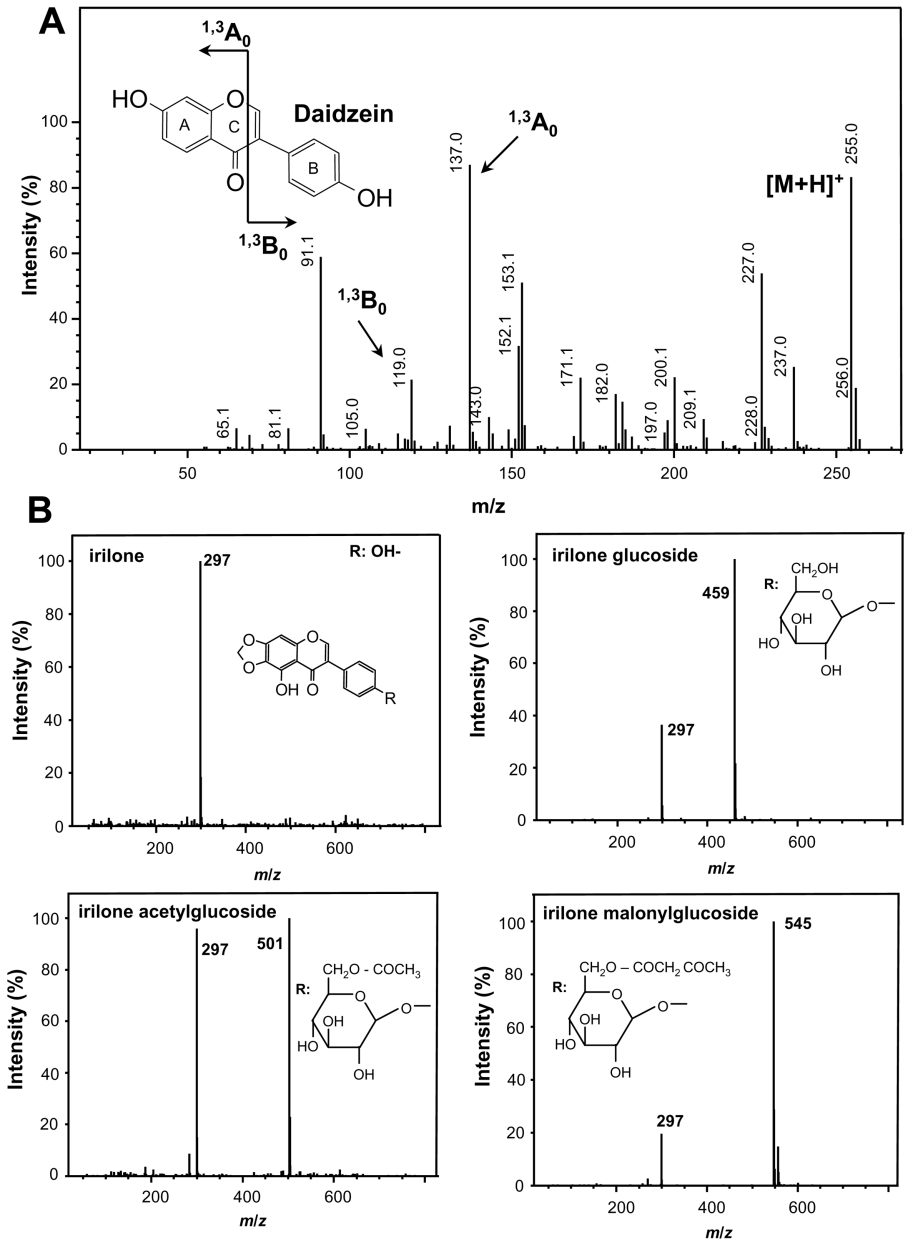


Figure 6. Mass spectrometry of (A) daidzein (retro-Diels-Alder reaction scheme is shown) and (B) irilone and its β-glucosides. Electrospray-quadrupole MS was used. Parameters: mobile phase 0.2% acetic acid and acetonitrile, gas temperature: 300°C, drying gas flow 10 L/min, nebulizing gas 40 psi, capillary voltage 3.5 kV, scan 50–600 *m/z*, fragmentor voltage 220 V (for A) and 110 V (for B) was used. For other details see [29].

isoflavones profiles [68] and for many other applications (reviewed in [61, 65, 69]). Chromatography with quadrupole TOF MS was used for analysis of phytoestrogens and isoflavones in water samples. Detection limits of determined estrogens and isoflavones ranged from 5 to 30 ng/L [42].

Investment and proven costs for MS are much higher compare to the other detection techniques. The selectivity of classical spectrophotometric techniques could possibly be increased using derivatization agent that is selectively bound to the analyzed isoflavones.

In addition to the classical UV-Vis detection, fluorescence detection with a derivatization agent can be used. A native fluorescence of individual isoflavones that can be observed for puerarin and daidzein at pH 8–9 [70] was also utilized. The acidity of the mobile phase causes a problem, thus post-column addition of an alkaline buffer greatly increases the sensitivity of analyses (improvement of a sensitivity about 500–600 fold). The signal-to-noise ratio is 150 times better than for classical UV-Vis detection. Limits of quantification (LOQ) in rat plasma were 3.48 and 1.16 ng/mL for puerarin and daidzein, respectively. Due to the native fluorescence of isoflavones their derivatization by fluorescence agents is in many cases very limited.

Chemiluminescence techniques can be also used for sensitive analysis of isoflavones. Combination of chemiluminescence and flow injection analysis (FIA) was used

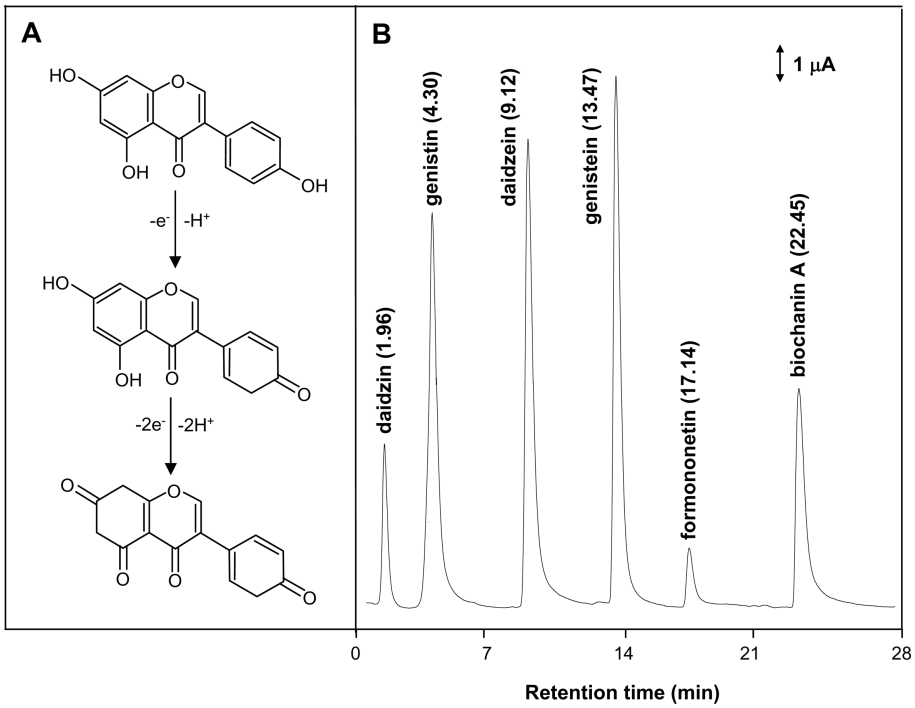


Figure 7. Electroanalysis of isoflavones. (A) Electrochemical oxidation of genistein. (B) HPLC–ED chromatogram of isoflavones. Parameters of measurement: Atlantis dC_{18} column (Waters, USA, 150 × 2.1 mm, 3.5 μm), mobile phase: acetonitrile and acetate buffer, pH 5.5 (solvent B), linear gradient elution: from 0 to 2 min 87% B, 22 min 60% B, 27 min 50% B, 31 min 45% B, 47 min 87% B, flow rate 0.4 mL/min, column and detector temperature: 35°C, injected volume: 5 μL, concentration of isoflavones: 10 μg/mL. CoulArray electrochemical detector was used (potential of working electrodes: 450 mV). For other details see [80].

for analysis of puerarin in different samples with limits of detection of 0.1 ng/mL. Using peristaltic pumps and a suitably designed FIA manifold a sample containing puerarin was mixed with luminol, KIO_4, and NaOH solution. After the reaction was complete, the mixture was injected into the reaction cell of a luminometer. Using the described technique, approx. 180 samples per hour could be analyzed at a flow rate of 2 mL/min [71]. Chemiluminiscence detection presents effective optical detection of selected isoflavones that can be used without any complicated separation and that can be applied in many clinical or food or agricultural labs or institutions.

4.4.2 Immunoanalysis

Immunochemical analysis is one of the very important techniques for fast identification of isoflavones in plant materials, foodstuffs, and pharmaceutical preparations. Initially developed techniques applying radioactive labeling (RIA: radioimmunoassay) [72] have today been replaced by techniques of enzyme-linked immunosorbent assay (ELISA) and their modifications [73–76].

In the case of ELISA it is necessary to prepare antibodies (monoclonal or polyclonal) against analyzed isoflavones or their conjugates (antigens) between isoflavones and a bovine serum albumin (BSA) or other high-molecular carrier (for details see [74]). Samples are transferred onto a microtitration plate and the antibodies obtained are bound to antigens present in the sample. After washing procedures, the amount of bound antibodies is quantified using secondary antibodies marked usually by peroxidase. After addition of a substrate of peroxidase and formation of suitable conditions for enzymatic reaction, the final product is detected. Its concentration corresponds to the amount of isoflavone determined in a sample.

ELISA was used for determination of biochanin A, daidzein, and genistein with limits of detection (LODs) that ranged from 1.1 up to 5.3 pg isoflavones per well [73]. Later ELISA was used for analysis of genistein and daidzein in foodstuffs and human body fluids (plasma and urine). The distribution (kinetic study with consumption of 100 mg of isoflavones) of daidzein and genistein in female plasma and urine was studied using ELISA [75]. Maximum daidzein and genistein concentration was detected after 6–8 h and after 10 h in plasma and in urine, respectively, after their oral administration.

A time-resolved fluoroimmunoassay (TR-FIA) method is another possibility for immuno-detection of isoflavones. The antibody is conjugated to a chelate of transition metal (Eu, Tb, Sm) that is transferred to a fluorescent marker in the course of an immunochemical reaction. Detection of isoflavones in homogenates of plant samples or their extracts, in foods, and in clinical samples is

possible using immunochemical techniques. Applications of immunoanalytical methods are common in clinical chemistry, mainly due to their simplicity and speed of the analyses (see review [77]).

4.4.3 Electrochemical methods

Since isoflavones are electroactive substances, many techniques employing electrochemical detection or combination of flow techniques and column separations with electrochemical detectors were developed. Electrodes manufactured from carbon and its different modifications were found suitable for possible measurement of oxidative signals of isoflavones (see genistein oxidation in Fig. 7A). Glassy carbon or carbon paste can also be used as electrode materials.

A glassy carbon electrode (GCE) was used for analysis of flavonoids and phenolic acids applying differential pulse voltammetry with limits of detection in units of μM [78] and for determination of total contents of isoflavones in soy samples [79]. Carbon paste electrode (CPE) was used for determination of daidzein and genistein [80]. Two oxidation peaks were observed (peak I around 0.4 V and peak II around 0.7 V). The peak I potential corresponds to oxidation of daidzein and genistein. The peaks found at more positive potentials are probably connected with adsorption processes of the oxidation products formed [80].

Adsorptive stripping voltammetry with carbon paste electrode [81] was used for analyses of selected flavonoids. Electrode material was prepared by mixing nujol or diphenyl ether with carbon powder and electrochemical detection performed under FIA conditions. Flavonoids were accumulated at the surface of carbon electrode at a working potential 0.2 V and subsequently the electrochemical scan was monitored from 0 up to 1 V. Oxidation signals of flavonoids were observed at 0.4 V and in the case of rutin well developed peaks were observed in the concentration interval 10 – 100 nM [81]. The technique was used for analyses of total contents of flavonols in wines, tea infusions, and juices [82].

Electrochemical detectors are also well suited for CE separations (see Section 4.3) and they can also be used in chromatographic separations. Detection limits of 51 fmol (21 pg) per injection were obtained using a coulometrical detector (in connection with RP-HPLC) at a working potential of 0.45 V (a chromatogram of 10 μg/mL of isoflavones is shown in Fig. 7B).

5 Methods for the study of isoflavone interactions with other bioactive compounds

The molecular principles of physiological effects of isoflavones are based not only on their estrogenic activity, but also on their interactions with a huge number of cell substrates. The main interests of scientists are focused on interactions of isoflavones with nucleic acids [83, 84], proteins [85, 86], and cell receptors [87]. We expect that a thorough knowledge of the interactions of isoflavones with other substances will shed light on their physiological activities and also resolve controversial aspects of their positive and/or negative influences on human health.

It was confirmed that isoflavones could interact with DNA and form complexes with bases present therein. Li *et al*. [84] studied interactions between guanine-rich telomeric DNA and daidzin by ESI-MS, polyacrylamide gel electrophoresis, and circular dichroism methods, and by using molecular simulation. Their results show that isoflavones can not only interact with DNA but can also stabilize its structure – as G-quadruplex, for example. Daidzin could serve as an anti-cancer compound inhibiting telomerase activity [84]. Interactions of quercetin and rutin with guanine and adenine in a single stranded chromosomal DNA were observed using square-wave voltammetry with a carbon electrode. Presumably the technique will be generally applicable for fast and sensitive detection of interactions of flavonoid substances and DNA [83].

In addition to DNA, isoflavones interact also with other biomacromolecules. Interaction of human serum albumin with genistein and other isoflavones was, for example, studied by spectrofluorimetry. The interaction of genistein with tryptophan residues in the protein molecules was confirmed [86]. Binding of genistein, biochanin A, formononetin, coumestrol, and others on human estrogen receptor α and β was studied using competition-binding assay [87].

Very important are also studies focused on the interaction of isoflavones with a cytoplasmic cellular membrane that is formed by a lipid bilayer. Adsorption of isoflavones on lipidic membranes, their influence on the peroxidation of lipids, and the integrity and permeability of cell membranes [88] have been mainly studied. The results should be applicable to studies of pharmacokinetic parameters of isoflavones present in drugs and in pharmaceutical preparations.

6 Isoflavones in agricultural research and food chemistry

A huge number of physiological effects of isoflavones on humans and other living organisms are well known (see above). Their biological activity could possibly be exploited in medical, pharmaceutical, and other areas. Some physiological disorders were observed in animals consuming plants and feeds containing high concentrations of isoflavones or enormous quantities of feeds con-

taining isoflavones (mainly reproductive dysfunctions arising from the estrogenic activity of isoflavones). Elimination of excessive synthesis of isoflavones, breeding of cultivars with reduced content of isoflavones, and/or strict monitoring of antinutritional substances in plant materials are of great importance in plant production.

The presence of isoflavones and phenolic acids was monitored in pea seeds (*Pisum sativum*) using the HPLC/UV-Vis technique. The content of isoflavones in the seed was reduced using special technological processes [89]. It was shown that storage conditions and suitable physical and chemical treatment could change the content of isoflavones in plant products. In contrast, some techniques of genetic engineering were developed to influence the content of isoflavones (daidzein, glycitein, genistein) in soybean seed on the basis of expression of maize C1 and R transcription factors that activated the phenylpropanoid biosynthetic pathway. The HPLC/UV-Vis method was used for identification of isoflavones after their extraction with methanol [90].

Foods and/or food supplements (functional foods – most commonly produced on the basis of legumes) are the most common source of isoflavones for human organisms*. The term functional foods was first introduced in Japan in the 1980s [59]. A functional food is at present defined as a foodstuff that produces a positive (beneficial) effect on one or more physiological functions of the human body or reduces the risk of disease (the role is not curative but preventive). In addition to soy and their products (*i.e.* tofu – soy cheese, sofu – fermented tofu, miso – soy rice), phytoestrogens and isoflavones were identified in a huge number of food products, *e.g.* kudzu (white starch powder from roots of *Pueraria lobata*), hops (*Humulus lupulus*), alcoholic drinks (beer, bourbon), and many other materials (fruits *etc.*) [91].

Analyses of secondary metabolites (and not only isoflavones) in various plants confirmed that the distribution of the substances in plant tissues is highly dependent upon ripening and growing conditions. Techniques of genetic engineering offer possibilities of producing plants with defined contents of isoflavones in near future. In this context it is necessary to consider not only the beneficial effects of isoflavones on human health, but also the fact that consumption of excessive amounts of isoflavones can give rise to numerous different health complaints [3].

7 Concluding remarks and outlook

Because the chemical structures and physico-chemical properties of isoflavones, and also other flavonoids, are very similar, their identification in plant matrices is very complicated. Recognition of a huge number of physiological effects on human health has also prompted developments in the area of instrumental methods of analysis of the substances. Improvements are expected mainly in techniques of sample preparation and methods of isolation – especially ASE, SFE, SPE, and micro-extraction techniques [92]. Monolithic columns, columns filled with sorbents of particle size <2 μm (separation times are greatly reduced by the small particle size), and also capillary columns will predominate in liquid chromatography.

Applications of various instrumental configurations of MS detectors should bring further progress in the detection of isoflavones and their identification in complicated plant matrices. And ESI continues to be most commonly applied ionization technique. NanoESI [93] and also recently introduced DESI (desorption-electrospray ionization) techniques appear to hold considerable promise. DESI represents a transition between ESI and MALDI and allows relatively sensitive analyses of biologically important substances under *in situ* conditions, thus minimizing the time required for pretreatment of samples [94].

Immunoanalytical techniques will find applications mainly in clinical practice in analyses of body fluids and in routine analyses of a large series of samples (mainly techniques with non-radioactive labeling – ELISA and TR-FIA) [95]. In addition, some papers have been published which describe successful applications of immunochemical techniques in the identification of isoflavones in different plant materials and food products.

Electrochemical detection of isoflavones is very commonly used in connection with electromigration separation techniques [96, 97]. Different constructions of electrochemical detectors have been recommended. Glassy carbon was identified as the most promising electrode materials. On the other hand, carbon paste electrodes (CPE) proved beneficial in a classical three-electrode voltammetric configuration. It is presumed that other materials will find applications in the near future and also serious miniaturization of detection cells and working electrodes will improve their performance. Nanomaterials (*i.e.* carbon nanotubes [98]) will also be utilized in their production.

Complete separation of the whole spectrum of isoflavones in several minutes and their detection in ng quantities in different plant materials, clinical samples, and foodstuffs is now possible using modern analytical methods. This fact is a key parameter in the precise control of quality of agricultural and food products.

* Special issues of *Anal. Bioanal. Chem.* Vol. 389 (No. 1) 2007 and *J. Sep. Sci.* Vol. 30 (No. 4) 2007 focuesed on the problematic topic of isoflavones and other phenolic substances in foods. The very latest information on analysis and physiological importance of isoflavones in human health can be found in a special issue *J. AOAC Int.* Vol. 89 (No. 4) 2006.

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