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Review

Review of the methods used in the determination of phytoestrogens☆

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Abstract

Interest in analytical methods for plant estrogens (phytoestrogens) has risen sharply in the past 10 years. In this review, we examine the existing analytical methods based on separations by gas–liquid chromatography, high-performance liquid chromatography and capillary electrophoresis in addition to methods of detection by ultraviolet absorption, fluorescence, electrochemical oxidation/reduction and mass spectrometry. These methods are compared with other methods of phytoestrogen analysis utilizing immunoassay approaches. The advantages and disadvantages of each of these methods are highlighted and potential areas for further development identified.

Keywords: Reviews; Phytoestrogens

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☆A wide range of units of mass (as g and mol) and concentration (as ppm, g/l and mol/l) have been used in the papers that are the subjects of this review. Accordingly, the units as quoted in each paper are given in the text of this article with corresponding alternative units to assist in data comparisons.

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1. Introduction

Phytoestrogens are members of classes of polyphenolic compounds synthesized by plants. They include isoflavones and other flavonoids, lignans, coumestanes, stilbenes and zearalenones. Over the past 15 years there has been a dramatic increase in the number of papers published annually on phytoestrogens, rising from five in 1985 to over 700 in 2000. The reason for this rapidly rising interest has been the association of many phytoestrogens and other members of the polyphenol family with specific diseases or toxicity-related issues [1,2]. This in turn has necessitated the development of new methods for the analysis and quantitative measurement of phytoestrogens.

In 1985, the principal method for the analysis of phytoestrogens (and other polyphenols) was gas chromatography (GC). Because of the lengthy procedure for purifying plant or physiological samples prior to analysis, investigators used ^{13}C- or ^{2}H-isotopically labeled phytoestrogens to correct for work up losses. This approach also required the use of a mass spectrometer coupled to the GC instrument (GC–MS) as well as chemical derivatization of the phytoestrogens. We have previously outlined the advantages and limitations of this method [3,4].

In this present review, we compare and contrast the analytical methods currently available to investigators for the analysis of phytoestrogens and their metabolites. A general comparison of these methods in terms of sensitivity, specificity and their advantages and limitations is listed in Table 1.

1.1. Chemistry of phytoestrogens

Phytoestrogens are found in plants and in many food products as glycosidic conjugates. In fermented foods, they are deconjugated to their aglycones. In soybeans, the predominant glycosidic conjugate is the 6″-*O*-malonyl-β-glucoside at the 7-position of the isoflavones (Fig. 1A). This conjugate undergoes decarboxylation to 6″-*O*-acetyl-β-glucoside (Fig. 1B) or hydrolysis to the β-glucoside (Fig. 1C) during preparation of soy foods [5]. The 7-*O*-glucosyl-glucoside of genistein (5,7,4′-trihydroxyisoflavone) is present in the tubers of *Apios americana*, a long time staple of the Eastern Native American [6]. However, the structure of this diglucoside of genistein has not yet been determined. C-Glycosides are also found, such as puerarin, 8-glucosyldaidzein (Fig. 1D). The common isoflavones genistein and daidzein are also found as the 4′-methyl ethers biochanin A and formononetin, respectively (Fig. 2A). A 6-methyl ether of 6,7,4′-trihydroxyisoflavone (glycitein) is present in soybeans (Fig. 2A). Equol (Fig. 2B) and *O*-desmethylangolensin (Fig. 2C) are common metabolites of daidzein and formononetin.

Lignans are polyphenolic compounds linked by a four-carbon bridge. Flaxseed is particularly enriched in the lignans matairesinol (Fig. 3A) and secoisolariciresinol (Fig. 3B) and these are converted by

Table 1
Comparison of analytical methods for analysis of phytoestrogens

Technique	Sensitivity	Specificity	Pros	Cons
GC–MS	50 fmol	High	High resolution	Complex work up
			Good for unknowns	Difficult chemistry
HPLC				
–UV (and DAD)	2 pmol	Moderate,	Good for soy food and conjugates	Low sensitivity
		better with DAD		Less specific
–Fluorescence	200 fmol	Good	Sensitive	Limited to fluorescent analytes
–ED (and array)	20 fmol	Better with detection array	Suitable for biological samples	Cannot determine novel compounds
–MS	1–500 fmol	High	Ease of use and sensitive	Limited chromatographic resolution
CE				
–UV (DAD)	50 fmol	Moderate,	High separation resolution	Limited injected sample volume
		better with DAD	Excellence mass sensitivity	Poor concentration sensitivity
–Fluorescence	1–5 fmol	Moderate	Sensitive	Limited fluorescent analytes
–ED	1–2 fmol	Moderate	Sensitive	Limited specificity
–MS	100 amol	High	Sensitive	Difficult interface
				Low resolution
UV and IR spectroscopy	NA	Fair	High throughput	Lack of specificity
MALDI-MS	100 fmol	High	High throughput	Lack of quantitation
Immunoassay	1–100 fmol	Good	High throughput	Cross reactivity

bacteria in the mammalian gastrointestinal tract to enterolactone (Fig. 3C) and enterodiol (Fig. 3D), respectively. Other members of the bioflavonoids that have estrogen-like properties include kaempferol, quercetin, apigenin (Fig. 4A), and 8-prenylnaringenin (Fig. 4B). Coumestanes, of which coumestrol is the most common (Fig. 4C), are present in plants such as *Alfa alfa*. *trans*-Resveratrol (Fig. 4D) is a stilbene present in red wine. Zearalenone (Fig. 4E) is found on, as opposed to being in, plants—it is an estrogen from contaminating fungi [7].

Once consumed, phytoestrogens undergo several metabolic transformations. Intestinal hydrolases remove the glycosidic groups allowing for rapid uptake of the aglycones and distribution to peripheral sites [8]. In the liver and certain other tissues, phytoestrogens are converted to β-glucuronides [9], sulfate esters [10], and methylated products [11]. Of the phase I reactions, hydroxylation by cytochrome P450 enzymes is a relatively minor event for isoflavones; however, demethylation (i.e., conversion of biochanin A to genistein or matairesinol to enterodiol) occurs readily [10–12]. The liver efficiently transfers isoflavones from the blood into bile [13], thereby returning the isoflavones to the gut. The slower hydrolysis of the phase II conjugates leads to a proportion of isoflavones coming into contact with the microflora of the large bowel in the second intestinal passage. Reduction of the heterocyclic ring (for daidzein to dihydrodaizein and then to the isoflavan equol) occurs. In an alternative pathway, the heterocyclic ring can be cleaved thereby forming *O*-desmethylangolensin and 2-(4-hydroxyphenyl) propionic acid [9,14], as well as several other phenolic acids [15]. In summary, for the analyst phytoestrogens and their metabolites range from the hydrophobic biochanin A to the hydrophilic β-glucuronide and glycoside conjugates.

1.2. Analytical methods

These can be divided into two categories: those that involve preliminary chromatographic separation, GC, high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE); and those that do not, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), deconvolution UV and infrared (IR) spectroscopy, and immunoassay.

2. Chromatographic methods

Separation of phytoestrogens by chromatography is particularly useful since it allows for the de-

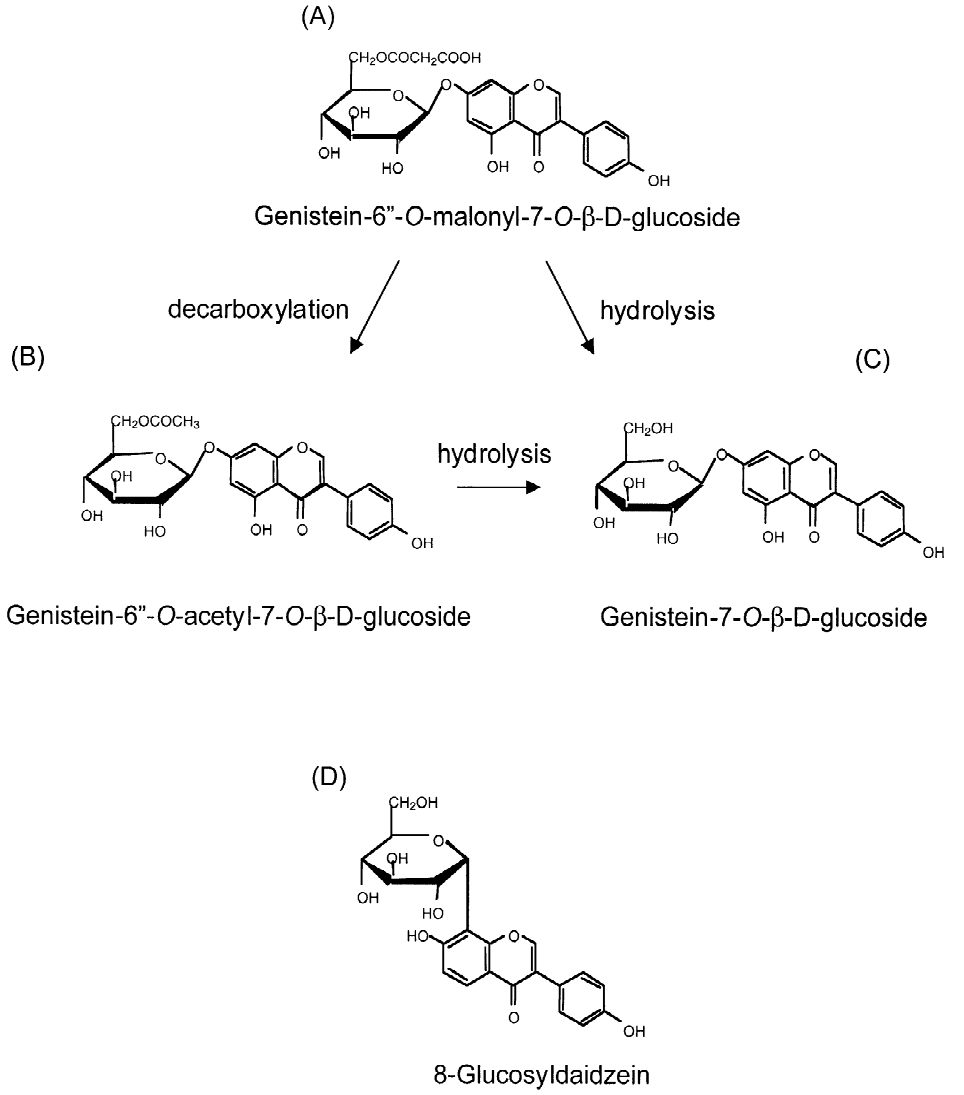

Fig. 1. Chemical structures of the glycoside conjugates of genistein. In unprocessed soybeans, the major glycoside is the 6″-*O*-malonyl-β-glucoside (A). Toasting of soy protein leads to decarboxylation and formation of the 6″-*O*-acetyl-β-glucoside (B). The hydrolysis that occurs in preparation of soymilk and tofu produces the de-esterified β-glucoside (C). Puerarin: 8-C-glucosyldaidzein (D) is present in the Kudzu.

termination of the physical properties of each phytoestrogen or phytoestrogen metabolite. This permits their identification, confirmation and/or quantitation. The choice of which chromatographic method to use depends on the sensitivity that is necessary, the complexity of the biological matrix as it relates to the time spent working up the samples prior to analysis, the chromatographic resolution required, and the expense. The attributes of each technique are discussed in the following sections and are summarized in Table 1.

A second important aspect of the analysis of phytoestrogens is whether or not to measure them in the conjugated or unconjugated form. For the most part, foods contain glycosidic conjugates—the exceptions are fermented foods. Physiologic fluids and tissue generally consist of β-glucuronide and sulfate esters of phytoestrogens. Although the concentra-

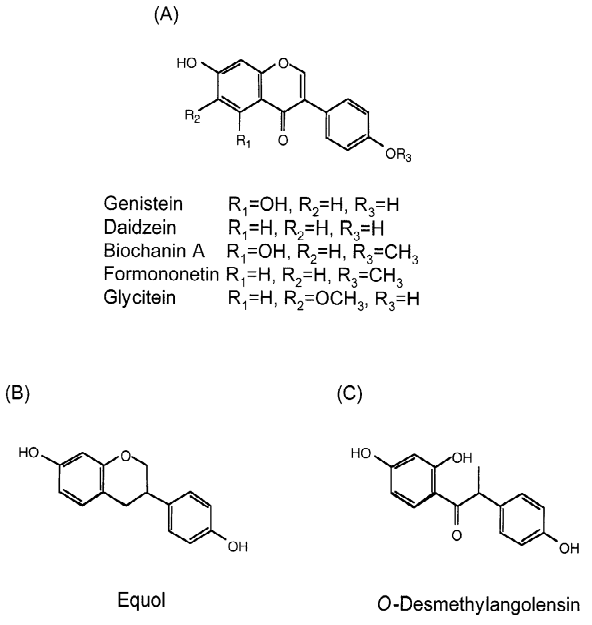

Fig. 2. Chemical structures of the isoflavones genistein, daidzein, biochanin A, formononetin, and glycitein (A). Equol (B) and *O*-desmethylangolensin (C) are common metabolites of isoflavones.

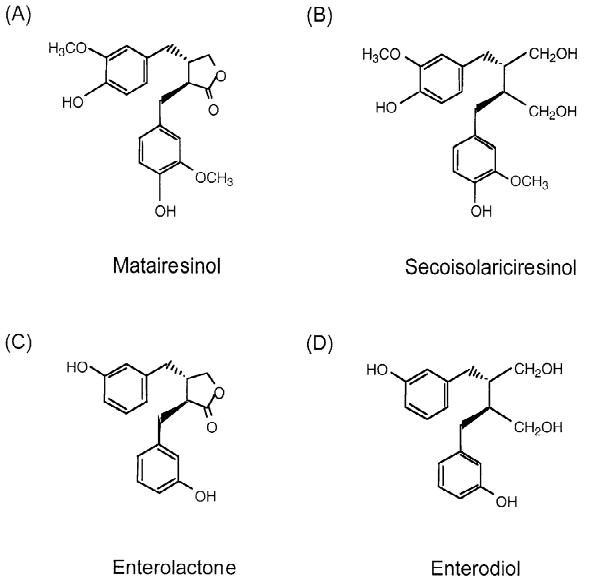

Fig. 3. Chemical structures of the lignans. Matairesinol (A) and secoisolariciresinol (B) are lignans in flax seed. Enterolactone (C) and enterodiol (D) are formed by bacteria in the gut.

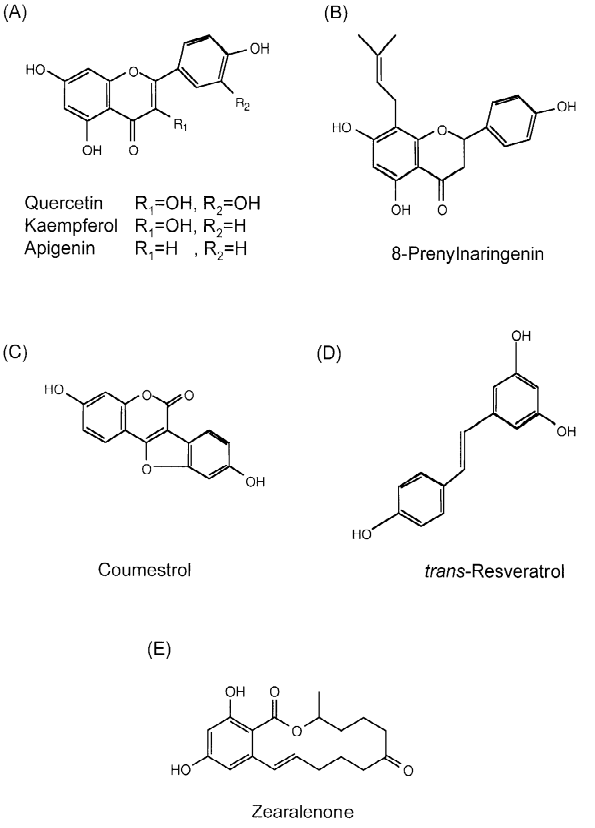

Fig. 4. Chemical structures of some of other phytoestrogens. Flavonoids, kaempferol, quercetin, apigenin (A), and 8-prenylnaringenin (B). Coumestrol (C) is found in low concentrations in many plants. *trans*-Resveratrol (D) is found in red wine and onion. Zearalenone (E) is a fungal phytoestrogen that contaminates wheat and silage products.

tions of the unconjugated phytoestrogens may only represent 1–5% of the total phytoestrogens and be in the low n*M* range, they are the forms that enter tissues. Therefore, there are occasions where selective measurements of the unconjugated phytoestrogens are warranted.

To carry out quantitative measurements of phytoestrogens using chromatographic methods, it has proved necessary to include internal standards to correct for unknown losses during the procedure used (Table 2). These standards range from deuterated (^{2}H) or carbon-13 (^{13}C) labeled stable isotope forms of the phytoestrogen of interest, or compounds with similar chemical structure and properties that are not naturally present in the sample to be studied.

Table 2
Internal standards used in phytoestrogens analyses

Internal standard	Method	Refs.
Deuterated isoflavones and lignans	GC–MS analysis of isoflavonoids	[36,32]
Deuterated daidzein	GC–MS analysis of glycitein	[33]
Kaempferol	GC–MS analysis of 8-prenylnaringenin	[31]
Fluorescein	HPLC analyses of phytoestrogens	[76]
Flavone		[45]
p-Hydroxybenzoic acid	HPLC analysis for isoflavones	[95]
Apigenin	LC–MS analysis for isoflavones	[4,50,81]
Deuterated genistein and daidzein		[73]
Biochanin A		[3,4]
Phenolphthalein β-glucuronide		
4-Methylumbelliferone sulfate		
1-Methyl-3-isobutylxanthin	CZE–UV analysis of isoflavones	[113,116]

GC–MS methods, because of their extensive set of work up steps, have the greatest need for internal standards. However, even with ^{2}H- or ^{13}C-labeled phytoestrogen standards, no account is taken of the losses that occur during initial extraction and hydrolysis prior to addition of the internal standard. For LC-based techniques, glycoside, β-glucuronide and sulfate esters of suitable analogous compounds have been used to correct for extraction and incomplete hydrolysis.

2.1. Gas chromatography–mass spectrometry

This method has been the mainstay of phytoestrogen analysis for the past 20 years, particularly for the low concentrations of phytoestrogens in blood, feces and urine [16–28]. Although providing very high chromatographic resolution, it is a very labor-intensive method. A clean up procedure is usually carried out to remove co-extracted compounds from the biological matrix that otherwise would be detrimental to the final analysis. A simple procedure for the isolation of isoflavones from aqueous sample (human urine) is based on liquid–liquid extraction using a ChemElut column (containing diatomaceous material that absorbs and retains water from the matrix) connected on-line with a Florisil cartridge by a PTFE stopcock for effective purification [29,30]. The high surface area of cartridge filling allows efficient free interaction between the sample and an organic extraction solvent. Extraction does not require vacuum but is carried out using gravity only. This procedure was also extended to the isolation of the hop-derived phytoestrogen, 8-prenylnaringenin, from beer, thereby using methanol for elution from the SPE column and diethyl ether–ethyl acetate (1:1, v/v) for desorption from the Florisil cartridge [31]. Because of the multi-step nature of the clean up procedure, it has large and sometimes variable losses, necessitating the use of stable isotope (^{2}H or ^{13}C) labeled internal phytoestrogen standards [19,20,22]. Adlercreutz et al. have used an isotope dilution GC–MS (ID–GC–MS) method for the detection of isoflavones (including daidzein and genistein) and lignans in human feces [32]. In this method, following the addition of deuterated internal standards for all compounds, the samples are extracted and purified in several ion-exchange chromatographic steps prior to GC–MS analysis. The combination of cation and anion-exchange column gives better purification of the phytoestrogens in fecal samples. The limit of detection of compounds (unconjugated fecal lignans and isoflavones in omnivorous and vegetarian women) in feces is about 1 to 14 nmol/24 h. Heinonen et al. [33] described the presence of the isoflavone metabolites dihydrodaidzein, dihydrogenistein, 6′-OH-*O*-desmethylangolensin and *cis*-4-OH-equol in human urine by GC–MS using authentic reference compounds. These authors used absorption chromatography on Sephadex LH-20.

Common dietary phytoestrogens contain at least one hydroxyl group in their structure making it difficult for their analysis by GC–MS without derivatization. Formation of derivatives is needed in GC to increase the volatility of an analyte (especially

for those possessing a hydroxyl, carboxyl or amino group), to improve its thermal stability, and to enhance the sensitivity or selectivity of the detection. It usually involves the production of trimethylsilyl ether (TMS) derivatives with *N,O*-bis-(trimethylsilyl)-trifluoroacetamide containing 1% trimethylchlorosilane. Other silylating reagents, such as pyridine–hexamethyldisilazane–trimethylchlorosilane have also been reported [33]. In some publications, authors reported that it was difficult to quantitatively derivatize polyhydroxylated isoflavones such as genistein [34]. TMS derivatives are analyzed using a nonpolar capillary column and a linear temperature gradient.

GC–MS in the electron impact (EI) ionization mode has been widely used in phytoestrogen analysis. A selected ion monitoring mode has been applied to the detection of isoflavones and their metabolites [30,31,35]. EI ionization of TMS derivatives of the isoflavone metabolites generally gives mass spectra containing intense peaks due to their molecular ion or a $[M-15]^+$ ion (loss of CH_3 radical) [30]. EI mass spectra can thus be used to determine the molecular mass of the metabolites.

Phytoestrogens, particularly isoflavones exist in biological fluids (serum, plasma, and urine) as glucuronide and sulfate conjugates and, in much small amounts, as their aglycones. For the determination of the total isoflavone content, a hydrolysis step is required. This can be accomplished using a mixed β-glucuronidase/sulfatase preparation from *Helix pomatia*—the resulting unconjugated isoflavones are extracted by a solvent partition technique [4]. This procedure has the advantage that most of the electrolytes in the sample are left behind in the aqueous phase, which is not the case for SPE. Quantitative determination of phytoestrogens in plant-derived foods by ID–GC–MS has been described [36]. The main methodological problem was to achieve complete hydrolysis of all glycosides of lignans and isoflavones without too much loss. Because the deuterated internal standards for the lignans are not stable during acid hydrolysis, they had to be added after that step. A three-step hydrolysis approach—rehydration with water, followed by acid and enzymatic hydrolysis—was applied to convert the diphenolic lignan glycosides into their aglycones. Similarly, lignans and isoflavones in plasma of omnivorous and vegetarian women were analyzed by ID–GC–MS. The diphenols may be measured at concentrations as low as 0.1 n*M* (0.03 ng/ml) [20]. Quantitative GC analysis of lignans and isoflavones was also carried out in plasma and prostatic fluid samples in men from Portugal, Hong Kong, and the UK [37]. In this study, higher levels of the isoflavones, equol and daidzein, were found in plasma and prostatic fluid in the samples from Asian men, compared with European men. High concentrations of equol (3270 ng/ml; 13.5 μ*M*) and daidzein (532 ng/ml; 2.1 μ*M*) were found in a sample of prostatic fluid from the Asian men. In general, the mean plasma concentrations of enterodiol from three centers were similar, at 6.2, 3.9 and 3.9 ng/ml (13–31 n*M*) in samples from Hong Kong, Portugal and the UK, respectively. The isoflavones from soya, which are present in higher concentrations in the prostatic fluid of Asian men, may be protective against prostate cancer.

GC–MS has been used to study the oxidative metabolism of the major soy isoflavones daidzein and genistein and lignans in vitro and in vivo [38–41]. Hepatic microsomal metabolism of the mammalian lignans enterolactone and enterodiol yielded 12 metabolites, six of which carry an additional hydroxy group in the aromatic rings and six in the aliphatic moiety [38]. Enterodiol is metabolized by hepatic microsomes from aroclor-treated male rats to three aromatic and four aliphatic ring monohydroxylated metabolites. Further work on metabolic fate of enterodiol and enterolactone in vivo was studied in rat bile and urine [39]. GC–MS analysis of the rat biliary metabolites of enterodiol indicated three metabolites of the aromatic hydroxylation type and two of aliphatic hydroxylation type (Fig. 5), while enterolactone provided six products of aromatic and five of aliphatic hydroxylation (Fig. 6).

Daidzein and genistein are metabolized by cytochrome P450 enzymes in vitro [40]. The monohydroxylated daidzein and genistein metabolites are not the endproducts of metabolic oxidation but are themselves substrates for the cytochrome P450 monooxygenase system. The oxidized daidzein metabolites were identified as 6,7,4′-trihydroxyisoflavone, 7,8,4′-trihydroxyisoflavone, 5,6,7′,4′-tetrahydroxyisoflavone and 6,7,3′,4′-tetrahydroxyisoflavone. Two monohydroxylated genistein metabolites

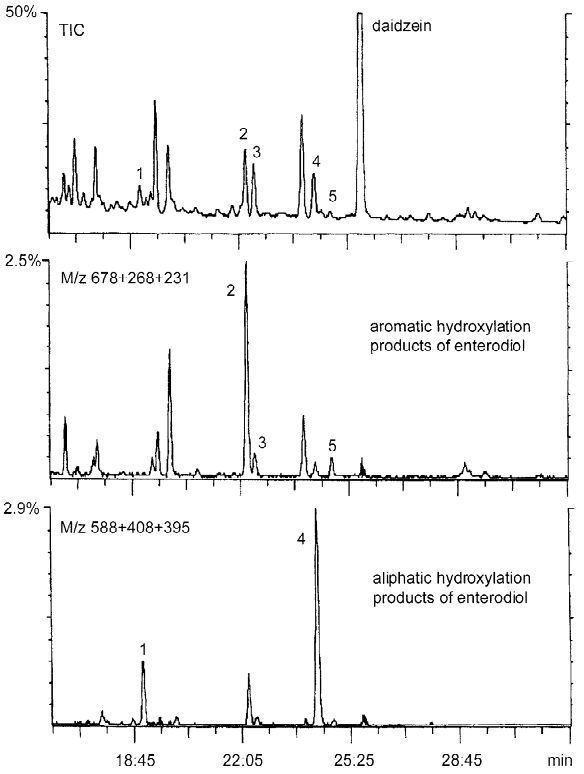

Fig. 5. Capillary GC–MS analysis of bile from enterodiol-dosed rats (HPLC fraction 1 after trimethylsilylation): (top) total ion current (TIC); (middle) ion current reconstructed from three typical ions of aromatic monohydroxylation products of enterodiol; (bottom) ion current reconstructed from three typical ions of aliphatic monohydroxylation products of enterodiol. The numbered peaks represent enterodiol metabolites. From Ref. [39] with permission.

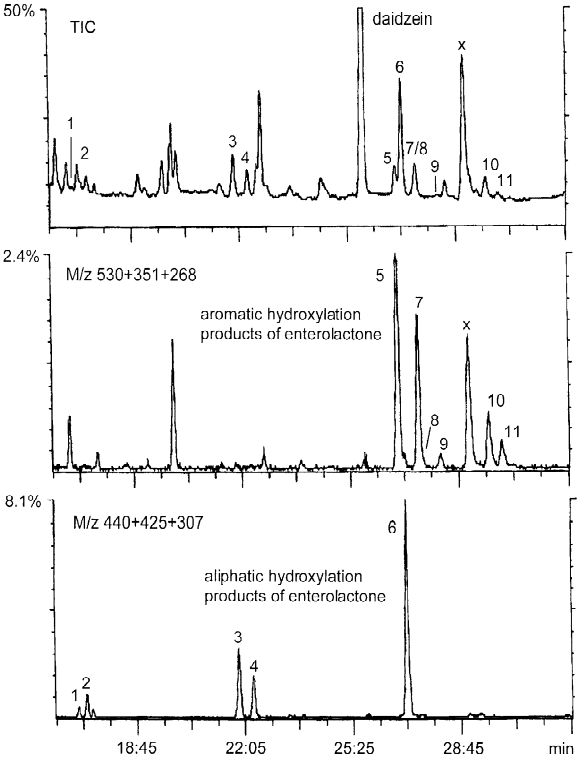

Fig. 6. Capillary GC–MS analysis of bile from enterolactone-dosed rats (HPLC fraction 1 after trimethylsilylation): (top) total ion current (TIC); (middle) ion current reconstructed from three typical ions of aromatic hydroxylation products of enterolactone; (bottom) ion current reconstructed from three typical ions of aliphatic monohydroxylation products of enterolactone. The numbered peaks represent enterolactone metabolites. From Ref. [39] with permission.

were also identified by GC–MS. Using human microsomes, genistein has been shown to be metabolized by human microsomes to six hydroxylated products based on GC–MS analysis [41].

Daidzein is an isoflavone with a low affinity for the estrogen receptor, but is consumed in relatively high doses (10–50 mg/day) by humans who eat soy products. Bayer et al. [42] have studied the disposition and biotransformation of daidzein in rats fed a diet low in isoflavone content. GC–MS analysis of the daidzein metabolites in feces revealed the presence of daidzein as a major peak, along with equol and *O*-desmethylangolensin, known daidzein metabolites in humans; on the other hand, there was no 4-hydroxybenzoic acid or 2-(4-hydroxyphenyl)-propionic acid, which potentially could be formed by reductive ring cleavage of daidzein in rats, analogous to the biotransformation of the related isoflavone genistein [9]. This paper also reports an interesting observation with regard to the gender difference in the formation of daidzein-sulfate—it is the predominant daidzein-conjugate excreted in males but is only a minor product excreted in female rats, a consequence of the sex difference in the expression of specific phenol sulfotransferases in rat liver [43].

2.2. High-performance liquid chromatography

Separation by HPLC obviates the need for derivatization of phytoestrogens and has been used

extensively with UV and/or fluorescence detection. The weakness of these detection methods is their non-specificity leading to the possibility of sample matrix interference. In general, the mobile phases employed with reversed-phase HPLC columns have been acetonitrile and/or methanol in combination with water containing small amounts of an acid. Details about the solvent systems and HPLC column have been reviewed by Merken and Beecher [44]. Sample preparation procedures for the analysis of phytoestrogens range from ''filter and inject'' (for urine) to solvent extraction (foods and tissues), hydrolysis of conjugates (foods and physiological samples), and sample clean up (SPE and solvent partition).

2.2.1. UV detection

All the phytoestrogens and their metabolites contain at least one aromatic ring. This means that they absorb UV light with a maximum wavelength (λ_{max}) in the range from 250 to 270 nm. Isoflavones with an intact heterocyclic ring absorb UV light much more strongly than their reduced (dihydrodaidzein and equol) or ring-opened counterparts (*O*-desmethylangolensin) [45]. Problems caused by differences in the wavelengths for maximum UV absorption by individual phyoestrogens and their metabolites can be dealt with by using diode array detection (DAD).

Franke and co-workers have contributed significantly to phytoestrogen analysis by reversed-phase HPLC [46–48]. This group developed methods for the efficient extraction and quantitative analysis of coumestrol, daidzein, genistein, formononetin, and biochanin A in legumes by reversed-phase HPLC–DAD using flavone as the internal standard (Table 2) [46]. In this approach, acid hydrolysis was carried out during extraction of foods to convert the various phytoestrogen conjugates into their respective aglycones. A solvent system consisting of A= acetonitrile, B=acetic acid–water (10:90, v/v); A–B (23:77, v/v) linearly to A–B (70:30) in 8 min followed by holding at A–B (23:77) for 12 min was used and the analytes were monitored at or very near their absorption maximum with dual-channel DAD at 260 and 342 nm. Coumestrol was detected at 342 nm.

Solid-phase extraction of urine was applied to phytoestrogen analysis followed by HPLC–UV–DAD detection [45]. Due to the possibility of chemical degradation of equol during acid hydrolysis, enzymatic hydrolysis (glucuronidase/sulfatase) was chosen. An additional merit of enzymatic hydrolysis is its simplicity in work up. The phytoestrogens were resolved using a step gradient elution and monitored at 260, 280, and 342 nm to achieve sensitive detection of all analytes. Franke and coworkers also used HPLC to analyze dietary phytoestrogens and their most abundant metabolites equol and *O*-desmethylangolensin in human fluids [47,48].

Most of the different classes of phytoestrogens and their metabolites are separated by reversed-phase HPLC using elution with a gradient of methanol or acetonitrile in an acidic (0.1–1% acetic, formic, or trifluoroacetic acids) or neutral (10 m*M* ammonium acetate or ammonium formate) solvent [44]. Our group has reported that the use of a neutral pH solvent provides better efficiency for chromatographic separation of a mixture of extracts of soy protein isolate and a soy germ food [6]. The 6″-*O*-malonyl-β-glycosides of isoflavones are more hydrophilic in neutral solvents than in acidic solvents and elute in front of the β-glycosides (Fig. 7). Complete class separation can be achieved in this neutral solvent system. The most often used columns are packed with reversed-phase C_{18} column material. However, C_8 reversed-phase columns are also useful for sepa-

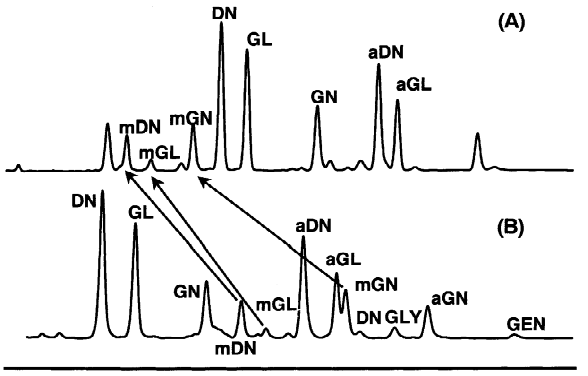

Fig. 7. The effect of changing the pH of the mobile phase during reversed-phase HPLC of isoflavones. A mixture of extracts of soy protein isolate and soy germ food were analyzed using an acetonitrile gradient in 10 m*M* ammonium acetate, pH 7.0 (A) and 0.1% trifluoroacetic acid (B). The malonyl esters of the isoflavones shifted from the middle of the chromatography (in B) to the front (in A). From Ref. [6] with permission.

ration of aglycones and glycosides of isoflavones [3]. There is a report on using isocratic HPLC–UV on a phenyl reversed-phase column and acetonitrile–water (33:67, v/v) as eluent to separate and quantitate isoflavones and coumestrol in soybeans [49].

Recently, Griffith and Collison presented an improved technique for extraction and HPLC analysis of isoflavones from food systems [50]. This method utilized acetonitrile–water–dimethyl sulfoxide (DMSO) extraction without acidification and used apigenin as an internal standard. Extracted materials were analyzed without further purification by gradient HPLC separation on a C_{18} reversed-phase column. It was effective enough to separate unexpected minor forms of malonyl and acetyl isoflavones in extracts of soy proteins and in pure isoflavone standard preparations.

2.2.2. Fluorescence detection

Detection based on fluorescence is generally more sensitive than UV absorption, due to the nature of detection geometry. Fluorescence is measured against a nearly zero background, whereas UV absorption is determined from decreases in the incident light source. It should be noted that the number of phytoestrogens that are naturally fluorescent is quite limited. However, this means that fluorescence detection can provide better selectivity in addition to better sensitivity compared to UV detection.

Wang et al. [51] reported a HPLC method for analysis of phytoestrogens in soybean and its processed products with both UV detection and fluorescence detection in series. UV absorption was monitored at 254 nm and fluorescence detection at 418 nm following excitation at 365 nm. Among five phytoestrogen standards detected by UV detection, only daidzein, formononetin and coumestrol were detected by fluorescence—genistein and biochanin A had no fluorescence response by this method. Coumestrol showed much stronger fluorescence response than by UV. The limit of detection for coumestrol by fluorescence was 0.5 ppm (mg/kg), whereas for isoflavones by UV it was 2 ppm (mg/kg). De Rijke et al. [52] also reported determination of 10 major isoflavones present in red clover by HPLC using fluorescence detection (excitation at 250 nm, emission at 418 nm), as well as by UV and MS methods. Only formononetin, its glycoside and 6″-*O*-malonyl-β-glucoside were detected by fluorescence. Daidzein, in this case, could not be measured by fluorescence detection due to the use of different excitation light source, 250 nm, instead of 365 nm mentioned above [51].

In order to take advantages of the sensitivity and specificity provided by fluorescence over UV, post-column derivatization of non-fluorescent flavonols has been applied [53]. Hollman et al. [54] reported a systematic study on the post-column reaction condition of flavonols and phytoestrogens with aluminum for fluorescence detection. Of the flavonoids, only flavonols that contained a free 3-hydroxyl and 4-keto oxygen binding site, such as quercetin and kaempferol, form fluorescent complexes with Al^{3+}. This method has a detection limit of 0.15 ng/ml (0.5 n*M*) for quercetin and 0.05 ng/ml (0.18 n*M*) for kaempferol, thus improving the limit of detection of quercetin 300-fold as compared to UV detection [55]. It remains to be seen if this method is suitable for phytoestrogens since they in general do not have a hydroxyl group in their heterocyclic ring.

2.2.3. Electrochemical detection

ED is a particularly useful method for determination of electroactive compounds, such as phenols, with better sensitivity than UV detection for HPLC applications [56–58]. The phytoestrogens, which contain phenolic groups, are generally electroactive and can be detected by ED. As early as the mid-1980s, HPLC with ED had been used to analyze isoflavones and other phytoestrogens presented in soybeans [59,60]. Setchell et al. [60] described a simple isocratic reversed-phase HPLC system, with ED, for the rapid separation of the phytoestrogens daidzein, genistein, coumestrol, formononetin, and biochanin A. In this study, the optimum potential for the simultaneous sensitive detection of coumestrol, genistein, and daidzein was +0.75 V, and the detection limits were 5 pg (19 fmol), 10 pg (37 fmol), and 15 pg (59 fmol) injected, respectively. Detection of formononetin and biochanin A required an operating potential above 1.2 V. At this detection potential, baseline stability was a problem, due to electrooxidation of impurities present in the mobile phase.

In studies from Franke's group [48,61], after enzymatic hydrolysis and extraction of isoflavones from food and human fluid samples, extracts were monitored simultaneously by DAD at 260 nm and 280 nm and coulometrically at +500 mV during the entire HPLC separation. In this study, ED further increased sensitivity for most analytes by a factor of 2 to 6 compared to DAD. However, the limits of detection from ED reported here were higher than those reported by Setchell et al. [60].

Since differences in the electroactive moiety on analogous structures can lead to characteristic differences in their voltammetric behavior, the use of a series of coulometric electrodes (coulometric array) with different potentials can provide selectivity for monitoring HPLC separation. Electrochemical array detection extends the resolution capabilities for co-eluted compounds whose oxidation or reduction potentials differ by as little as 60 mV [62]. Gamache et al. [63] described a gradient HPLC system with electrochemical array detection of up to 16 ED channels for analysis of phenolic and flavonoid compounds in juices. Twenty-seven standard compounds were resolved in two dimensions (chromatographically and voltammetrically) from a single HPLC run. Similar systems with eight ED channels were reported by Gamache and co-workers [64,65] for analysis of phytoestrogens in plasma, tissue, and urine samples. Twelve polyphenolic standards, such as coumestrol, genistein, equol and quercetin, were analyzed using this system with limits of detection of 5–50 pg (20–200 fmol).

As an additional benefit to the use of coulometric array for HPLC, it was claimed that the on-line generation of a voltammogram for each electroactive components could be used qualitatively to examine peak purity and aid in structural identification of unknowns. Nurmi and Adlercreutz [66] also described a HPLC system with electrochemical array detection for profiling phytoestrogens in plasma. A longer gradient elution was used to obtain high separation resolution with improved chromatographic repeatability and stability. Thirteen phytoestrogen standards were used to validate this method with limits of detection from 3.4 pg (9 fmol) for seco-isolariciresinol to 40.3 pg (149 fmol) for genistein: six of these 13 compounds were found in non-supplemented plasma, as shown in Fig. 8. This

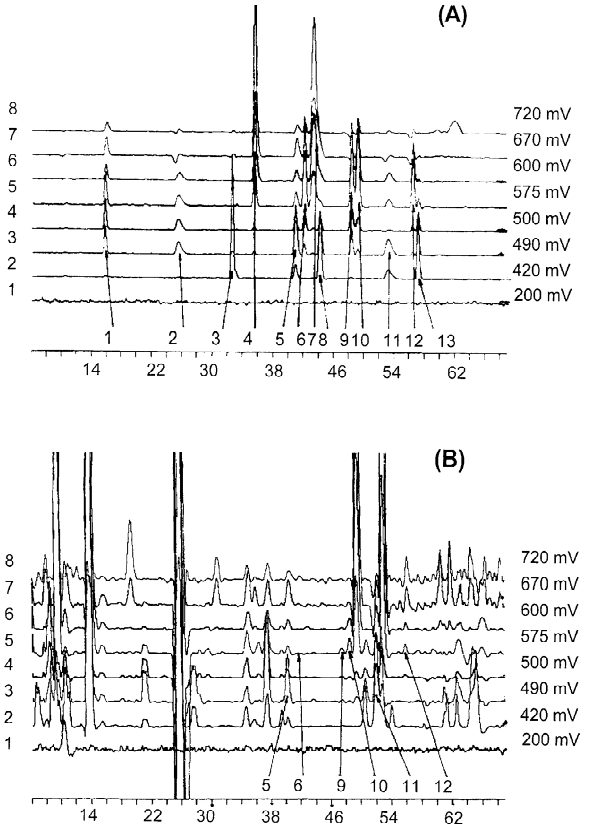

Fig. 8. Multichannel chromatogram of a standard phytoestrogen mixture (A) and the control plasma (B) by HPLC–ED analysis. 1, Daidzein-7-*O*-glucoside; 2, genistein-7-*O*-glucoside; 3, seco-isolariciresinol; 4, dihydrodaidzein; 5, daidzein; 6, enterodiol; 7, dihydrogenistein; 8, matairesinol, 9, equol; 10, enterolactone; 11, genistein; 12, *O*-desmethylangolensin; 13, anhydro seco-isolariciresinol. Modified from Ref. [66] with permission.

method was recently applied to quantitative analyses of lignan precursors and the mammalian lignans enterolactone and enterodiol [67]. Applications of HPLC–ED techniques to analysis of quercetin [68] and *trans*-resveratrol [69,70], as well as determination of genistein and daidzein in soy-based foods and supplements [71,72], were also reported recently.

2.2.4. Mass spectrometry

We have previously reviewed the progress in the analysis of isoflavones by HPLC with mass spectrometry (LC–MS) [3,4,6]. We now extend our review to the analysis of phytoestrogens by LC–MS

and discuss the methods of ionization, the choice of the mass spectrometer and the different ion analysis methods.

In the 1980s, HPLC was successfully coupled to a quadrupole mass spectrometer via a thermospray ionization interface and was applied to the analysis of phytoestrogens by Setchell et al. [60]. However, this method had practical limitations in terms of robustness and stability of the ion source. It has largely been superseded by the widely used atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) interfaces. These interfaces are highly sensitive, show greater ionization stability and have been the method of choice for isoflavone analysis, especially for clinical studies of isoflavone metabolites in animals and humans [3]. It should be noted that for ESI and APCI the use of trifluoroacetic acid as a HPLC mobile phase modifier causes ion suppression. Acetic acid or formic acid or their ammonium salts should be used as alternatives.

Much of the work using LC–MS since 1994 has focused on mass spectrometers with quadrupole detectors. Single quadrupole detectors are not particularly useful because of their low mass resolution and inability to carry out ion fragmentation. A more typical configuration is the triple quadrupole detector in which the phytoestrogen molecular ion is isolated by the first quadrupole, then subjected to collision-induced dissociation (CID), and the daughter fragment ions analyzed in the third quadrupole. This produces a MS–MS spectrum, very useful for confirming the identity of the phytoestrogen. By selecting unique daughter ions for each phytoestrogen, multiple reaction ion monitoring (MRM) can be developed as a highly sensitive, quantitative method of analysis.

Recently, application of the ion trap detector to the LC–MS analysis of phytoestrogens has been reported [50]. This method has several advantages: (1) it is very sensitive since ions can be accumulated in the ion trap, (2) the ion trap permits multiple sequential experiments with a given starting molecular ion—MS^n, and (3) the commercially available instruments are about half the price of their triple quadrupole counterparts. However, the fragmentation pathways in a quadrupole ion trap are different from those in a triple quadrupole instrument. Neither the ion trap nor the triple quadrupole instruments yield high mass accuracy, particularly in the MS–MS mode. This problem can be overcome by either using a hybrid quadrupole orthogonal time-of-flight mass spectrometer (Q-TOF-MS) that can deliver mass accuracies of 10–20 ppm in the MS–MS mode, or a Fourier transform-ion cyclotron resonance mass spectrometer that provides mass accuracies better than 1 ppm in both the MS and MS–MS modes.

Isoflavones are present in biological fluids as glucuronides and sulfate conjugates and in very small amounts as their aglycones. The sample preparation procedures include hydrolysis, followed by solvent partitioning. SPE is also widely used for qualitative analysis. In general, analysis of isoflavones and other phytoestrogens in biological samples are carried out on a reversed-phase column and the mobile phase is acetonitrile–water in 10 m*M* ammonium acetate. There is a report of using ammonium formate at pH 4.0 as an eluent [52].

The development and validation of analytical methods based on LC–ESI-MS for the use in determining blood isoflavones in rats has been published [73]. Analysis of rat blood using LC–ESI-MS showed that the genistein 7-β-glucuronide was the major form and the 4′-isomer was the minor form. The method uses serum/plasma deproteination and liquid–liquid extraction that was followed by solvent evaporation and sample dilution. A modified method that obviates the need for protein precipitation, extraction, and solvent removal was developed by Doerge et al. [74]. Genistein and daidzein were measured using on-line SPE and LC–ESI-MS detection in serum of rats and the limit of detection for isoflavones, based on the MS response was 20 n*M* (5.4 ng/ml). An improved method of detection of the isoflavone aglycones, genistein and daidzein has been reported using solid-phase microextraction–LC–ESI-MS by Satterfield et al. [75]. Isoflavones in 3 ml of urine sample was extracted using solid-phase microextraction with a Carbowax-templated resin fiber coating and on-line eluted into an LC–MS system for analysis. MS analysis was performed using an ion-trap MS with selected reaction monitoring. This method allowed rapid preconcentration of the analytes and provided good detection sensitivity. The lowest reported detectable levels of daidzein and genistein in urine were 25.4 (0.1 n*M*) and 2.70 pg/ml (0.01 n*M*), respectively.

Regarding the applications of LC–MS, we previously reported chemical modification of isoflavones in soyfoods during cooking and processing by reversed-phase HPLC–MS [76]. The result indicated that defatted soy flour that had not been heat-treated consisted mostly of 6′-*O*-malonyl-β-glucoside conjugates; in contrast, toasted soy flour contained large amount of 6′-*O*-acetyl-β-glucoside conjugates, formed by heat-induced decarboxylation of the malonate group to acetate. A microbore HPLC–ESI-MS positive ionization method for the determination of total daidzein and genistein in soy flour and baby food was developed by Barnes et al. [77]. They used API in the form of pneumatically assisted electrospray and the limit of detection was 0.2 mg/kg (0.2 ppm) for daidzein and 0.7 mg/kg (0.7 ppm) for genistein in the flour and food sample. In a recent analysis of isoflavones from soy food by reversed-phase LC–MS, an ion trap in the positive ion mode was used with multistage MS–MS [78]. In the case of malonyl, acetyl and glucoside forms of isoflavone, MS–MS gives the aglycone core, and MS^3 fragments can act as a recognizable finger print [50].

Interpretation of MS–MS spectra can be used in the identification of phytoestrogens. Kulling et al. [40] claimed that the location of the hydroxyl groups of the metabolites can be obtained from LC–MS with positive APCI-MS–MS using the base peak $[M+H]^+$ as well as fragment ions derived from the molecular ion by a retro Diels–Alder reaction. These ions can be used to determine the number of OH groups in the A-ring of the molecules. Fragment ions due to the loss of H_2O and CO were also observed in the MS–MS spectrum indicative of substituent positioning. Roberts-Kirchhoff et al. have also studied the metabolism of genistein by rat and human cytochrome P450s [78]. They claimed that the retro Diels–Alder decomposition product, a predominant ion in the CID spectra of all flavonoids obtained using quadrupole ion trap mass spectrometry, could be used to determine the number of substituents.

Cimino et al. [79] reported a LC–MS method to estimate urinary concentration of genistein and daidzein, and their sulfate and glucuronide conjugates in urine samples. In this study, isoflavones are separated using a gradient LC-method and detected by negative single ion monitoring on a MS system with a heated nebulizer APCI interface. They found 52±4 and 26±4% of genistein in rat urine as aglycone and sulfate conjugate, respectively, compared to 0.36 and 9%, respectively, in human urine. Likewise, Valentin-Blasini et al. used APCI-MS–MS for the measurement of seven phytoestrogens in human serum and urine [80]. This method uses enzymatic deconjugation of the phytoestrogen metabolites followed by SPE and reversed-phase HPLC. The method allows detection of isoflavone and lignans with a limit of detection in the low parts per billion range (ng/ml).

In our laboratory we have used enzymatic hydrolysis in the diluted physiological samples (plasma, serum or urine), followed by solvent extraction first to remove neutral lipids with hexane and then with ether to recover the phytoestrogen aglycones [81]. To control the hydrolysis and extraction steps, phenolphthalein β-glucuronide and 4-methylumbelliferone sulfate and apigenin, the flavonoid isomer of genistein, are added to each sample as internal standards (Table 2). This is comparable to the use of isotope dilution analysis mentioned earlier. MRM analysis permits the measurement of phytoestrogens with high sensitivity and specificity and without the need for chromatographic resolution. In fact, we have found that the analyses can be carried out rapidly under isocratic conditions (35% aqueous acetonitrile) in 2–3 min, enabling a complete analysis every 6 min. This technique greatly simplifies the analysis of phytoestrogens, particularly since it removes the necessity for gradient chromatography. This method has been applied to phytoestrogen analysis in several clinical studies [81–85].

The limit of detection using reversed-phase HPLC on a 10 cm×4.6 mm I.D. column operating at a flow-rate of 1 ml/min is 1–5 pmol injected on-column. To increase sensitivity the CID conditions can be optimized since the fragmentation of each phytoestrogen molecular ion occurs at different collision energies [86]. Further increases in sensitivity can be achieved by reducing the internal diameter of the reversed-phase column. A 300 μm I.D. column operates at a flow-rate of 2–4 μl/min—at this flow-rate, all the column eluate can be introduced into the ESI source (for conventional HPLC columns, only 25 μl/min out of the 1000 μl/min eluate—i.e., 2.5%—reaches the ESI source). Further sensitivity increases are possible if the analytical method is miniaturized. We have built columns in

quartz capillary tubing that operate at 200 nl/min [6,87]. At this flow-rate, the tip of the capillary where the spray is produced can be brought within 1–2 mm of the orifice in the interface of the mass spectrometer. The efficiency of ion transfer from the liquid phase to the gas phase and into the mass spectrometer is therefore much higher. Using this approach, we have developed an analytical method that has a limit of detection of 1 fmol—1000 times better than our current method. It is to be expected that the introduction of microfluidic methods for HPLC analysis in general will spur improvements in sensitivity.

The soybean and its products have been considered goitrogenic in humans and animals [88]. LC–APCI-MS analysis has shown that the aglycones genistein and daidzein are the components that inhibit thyroid peroxidase catalyzed reaction [89]. Analysis of a hop-based dietary supplement by LC–MS has been reported recently by Coldham and Sauer [90]. Results indicated that only hop-associated phytoestrogens (8-prenylnaringenin, 6-prenylnaringenin, 6,8-diprenylnaringenin, xanthohumol and isoxanthohumol) are found in the dietary supplements at significant concentrations. Ishii et al. identified the flavonoid glycoside naringin in human urine by LC–ESI-MS–MS [91]. According to this report, LC–ESI-MS analysis of naringin (4′,5,7-trihydroxyflavone-7-rhamnoglucoside) fraction showed an intense peak at m/z 598 $[M+NH_4]^+$ which on MS–MS analysis provided a base peak at m/z 273 (naringenin, $[M+H]^+$).

APCI-MS analysis of isoflavone malonylglucosides in *Trifolium pratense* L. (red clover) extract was reported using ammonium formate at pH 4.0 as an eluent [52]. Our group has recently analyzed isoflavones in soy and the American groundnut *Apios americana* using selected ion monitoring during an HPLC analysis [6].

LC–MS–MS analysis using a triple quadrupole mass spectrometer has been used for structural information. Examination of the MS–MS spectra of genistein and daidzein indicated that the product ion m/z 133 is diagnostic for these isoflavones, but not for their flavonoid isomers, such as apigenin [4]. A tandem mass spectrometric study of some isoflavones and flavonoids has been recently reported by Hughes et al. and identification and measurement of genistein in wood pulp in untreated waste water and in treated effluent from a wood pulp mill has been done [92].

Reports on the chemical structure of flavones and isoflavones in crude mixtures by tandem mass spectrometry are being published in various journals [93–96]. Very recently, a low energy LC–CID-MS–MS procedure has been studied by Waridel et al. [93] for the on-line differentiation of 6-C- and 8-C-glycosides flavonoid isomers, under typical LC–APCI-MS conditions, on two types of instruments; a Q-TOF and an ion-trap. The MS–MS spectra obtained in the Q-TOF and ion trap are different, a probably reflection of their individual collision processes.

A paper by Borges et al. [94] on the structural analysis of flavonoids and flavonoids-*O*-glycoside from the ethanolic extract *Genista tenera* is worth mentioning. These investigators isolated two flavones, three isoflavones and one 7-*O*-glycosyl isoflavone from the ethanolic extract and their structures were characterized by liquid secondary ion mass spectrometry in combination of high energy collision-induced dissociation and MS–MS. Likewise, application of LC with UV and MS to monitor changes in profiles of isoflavones glycosides and free isoflavones in *Lupinus albus* L. has been reported by Bednarek et al. [95]. Further attempt to characterize flavonoids in extracts of fresh herbs by negative APCI-MS was made by Justesen [96]. He points out that negative APCI-MS can provide aglycone fragments by in-source fragmentation of glycosides and the resulting fragment ions can be further selected for fragmentation by CID MS–MS.

Though HPLC–MS has been used extensively for the analysis of phytoestrogens, it has certain limitations such as the inability to generate molecular ions for some members of the diverse class of flavonoids and ineffective isomer differentiation. Metal complexation with the use of a neutral auxillary ligand, 2,2′-bipyridine results in the formation of $[M^{II}(\text{flavonoid-H})\text{bpy}]^+$, ternary complexes with intensities that are 2 orders of magnitude greater than the corresponding protonated flavonoids and up to 1.5 orders of magnitude greater than the deprotonated flavonoids, based on CID patterns [97]. This report claims that the method is applicable for several

flavonoid glycosides, including isoflavones and may be suited to structural analysis of these compounds [98].

2.3. Capillary electrophoresis

CE is a relatively new separation technique compared to other chromatographic methods such as GC and HPLC. The theory of CE has been discussed in detail in many references [99–101]. Basically, separation by CE is a result of differences in electrophoretic mobilities of charged species in an electric field in small-diameter capillaries. The use of capillaries, of 50–100 μm I.D. and 150–360 μm O.D., offers advantages of rapid, high-resolution separation (up to 10^6 theoretical plates) with sample volumes in the nanoliter range, resulting in excellent mass detection limits (femto- to attomole of samples). Since first described in its modern format by Jorgenson and Lukcas in 1981 [102,103], CE has been developed into several modes, such as capillary zone electrophoresis (CZE) or micellar electrokinetic chromatography (MEKC). These techniques also have been applied to various classes of analytes, including macromolecules such as proteins, or small molecules such as drug metabolites [99–101]. Applications of CE in analysis of natural products, including tea components and resveratrol in wine have been reviewed [104–106].

CZE is the basic mode of CE techniques. Charged species are separated from each other in the capillary—all neutral species migrate at the same speed. Since most of the phytoestrogens are weak acids, alkaline buffers are used to ensure that the phenolic moiety is charged for electrophoretic separation. Borate buffer, which forms a charged complex with *cis*-diol moiety of the sugar ring, is also useful for analyses of β-glycosides of phytoestrogens. The influence of structure and buffer composition on electrophoretic behavior of flavonoids has been discussed in several studies [107–110].

MEKC, a modified CE technique, is performed by adding surfactants, such as sodium dodecyl sulfate (SDS), at levels above their critical micellar concentration in the running buffer. The surfactants form charged micelles and migrate in CE capillary under the electrical field, similar to all charged species. The analytes, both neutral and ionic species, are partitioning between the micelle and running buffer, which contributes additional selectivity to the separation. Therefore, the micelle is referred to as a pseudo-stationary phase, similar to the stationary phase in LC separation. MEKC has been extensively applied to separate various compounds including neutral and hydrophobic species [111,112].

2.3.1. UV detection

The instrumentation format of CE is similar to HPLC—therefore most detection methods used in HPLC can be adapted to monitor CE separations. Shihabi et al. described the basic CZE method for analysis of isoflavones and coumestrol isolated from plant extracts with UV detection at 214 nm [113]. The optimum separation occurred in the pH range of 8.5–8.8 with 200 m*M* borate buffer. Wang et al. also reported a CZE method with UV detection at 200 nm for analysis of phytoestrogens present in a Chinese herb, *Puerariae radix* [114]. Effects of pH and borate concentration on separation efficiency were studied. Results showed that migration time and separation resolution was increased while higher pH and borate concentrations were used in the running buffer. Rapid separation of five phytoestrogens, including genistin, genistein, daidzin, daidzein, and biochanin, within 6 min was demonstrated with 20 m*M* borate buffer at pH 10.1. Detection limits with UV detection were comparable at levels of few μg per ml (~10 μ*M*).

Diode array detection was also used to monitor CE separation of phytoestrogens. A CZE–DAD system with alkaline borate buffer (150 m*M*, pH 10.5) was used by Aussenac et al. to determine the isoflavones in soybean seeds to study the effects of variety and environment [115]. The isoflavones of whole seeds were isolated and identified. Daidzin, genistin, their malonyl esters, and their aglycones were routinely detected, while other forms (glycitin, malonyl glycitin, and its aglucone) were present in small amounts. All isoflavones were separated with baseline resolution within 16 min. Mellenthin and Galensa compared the analyses of isoflavones using CZE and HPLC with DAD [116]. The resolution in CE separation was not as good as Aussenac et al. had previously reported, probably due to the less

basic borate buffer (200 m*M*, pH 8.6) used in this study. However, eight isoflavones extracted from a toasted soy flour, including daidzin, genistin, and their aglucones, 6″-*O*-malonyl and 6″-*O*-acetyl derivatives), were separated in 8 min, which is a shorter time than that required by most HPLC methods. The detection limit for the HPLC method was about 0.01–0.03 mg/l (40–120 n*M*) compared with the 0.1–0.5 mg/l (400–2000 n*M*) in CZE. The lower sensitivity for the CZE method is due to the difference in the light path length (11 mm for the HPLC detection cell, but only 50–75 μm in CZE). It should be noted that in CZE only about 10 nl of sample is consumed in the analysis (i.e., 4–20 fmol), whereas for a typical 10 μl phytoestrogen sample analyzed by HPLC–UV at the limits of detection, 400–1200 fmol would be consumed.

Huang and Hsieh [117] described a MEKC method with DAD for determination of puerarin, daidzein and other major components in the traditional Chinese medicinal preparation of Ge-Gen-Tang, which is a mixture of *Puerariae radix* and other crude herbs. Separation of all analytes was affected by pH and SDS concentration, and the optimum separation efficiency was reported using pH 9.0 borate buffer containing 20 m*M* SDS. The seven key analytes were sufficiently separated within 10 min. Limit of detections for daidzein and puerarin reported in this method were comparable to the previous result from CZE–DAD methods at few μg/ml (4–10 μ*M*) [114].

2.3.2. Fluorescence detection

Beekman et al. [118] reported determination of isoflavones, especially formononetin, in red clover using MEKC with a special mode of laser-induced fluorescence (LIF) detection. The native-fluorescent isoflavones, such as daidzein, formononetin, and coumestrol, were excited at 275 nm by a modified argon-ion laser and fluorescence emission spectra was on-line recorded by an intensified diode-array detector. Instrumentation of this system was described in another report [119]. Detection limits for the three standard analytes were reported between 0.1 and 0.4 μ*M* (25–100 ng/ml). Among three major peaks shown in the analysis of red clover extract with this MEKC–LIF system, only one was identified as formononetin by sample spiking and the matchup of emission spectra. The method provided not only the inherent selectivity of fluorescence detection, but also the on-line recording of emission spectra for further identification.

2.3.3. Electrochemical detection

ED has also been adapted for CE separation. However, it may be necessary to separate the electrochemical detector from the CE capillary, since high noise levels may occur when the detector is exposed to the high electric field used for CE. Approaches to adapt ED for CE have been reviewed [120]. Chen et al. described a CE–ED method for determination of rutin and quercetin in plants [121]. A 300 μm diameter carbon disc electrode was positioned opposite the outlet of the capillary and used as the working electrode. Daidzein, rutin, and quercetin were well separated within 10 min in a 40 cm length capillary in a 100 m*M* borate buffer at pH 9.0. The response was linear over 3 orders of magnitude with detection limits ranging from 0.19 μ*M* (53 ng/ml) to 0.43 μ*M* (120 ng/ml) for all compounds. A similar system was later used to determine puerarin, daidzein and rutin in a Chinese traditional medicine, *Pueraria radix*, by the same research group [122].

2.3.4. Mass spectrometry

Mass spectrometry has been shown to be an excellent detection method for CE separation with features of sensitivity, universal detection and selectivity with capability of providing structural information. The relatively low flow-rates of CE (<1 μl/min) compared to conventional HPLC (1 ml/min) make it much better suited to interface with MS through an ESI interface since the effluent can be introduced into MS without splitting. However, caution must be taken to maintain the CE separation efficiency and resolution while maintaining the electrical continuity for CE separation and ESI interfacing. The first CE–MS interface, using silver metal deposition onto the capillary terminus as the point for electrical contact, was reported by Olivares et al. in 1987 [123]. Development of other CE–MS interfaces and their applications have been described in many reviews [124,125].

Aramendia and co-workers [126,127] explored the use of on-line CE–MS for separation and characterization of selected isoflavones. A triaxial electrospray

probe was used to interface CE with a single-quadrupole MS operating in the negative-ion mode. The triaxial ESI incorporated a sheath tube allowing additional solvent (the make-up solution) to be transported to the probe tip and mixed coaxially with the sample flow at the end of the CE capillary before spraying. The make-up solution was used to supplement the CE flow by the extent required for ESI and to make electrical contact between CE buffer and the spray tip. CE separation of isoflavones was performed with the MS-compatible ammonium acetate buffer (at pH 9.0), instead of borate buffer used in other detection systems described before. Fast separation of genistein, daidzein, biochanin A, and isoliquiritigenin was achieved with baseline resolution; however, pseudobatigenin, formononetin and biochanin A co-migrated in this system. Although those co-migrated isoflavones could not be readily resolved using CE with UV detection, they were resolved by CE–MS, as shown in Fig. 9 [126]. Sensitivity of this system relied on many factors. Optimum analytical signal for this system was found when volatile buffers were used at the lowest possible concentration (10–25 m*M*); in fact, higher concentrations produced lower ionization efficiencies for the analytes during electrospray. Under optimum conditions and in selected ion recording mode, a limit of detection of about 100 attomole, equivalent to 6–7 n*M* in solution, for almost all the isoflavones was reported. This was 100 times better than working in the scan mode (~10 fmol; 1 μ*M*) [127].

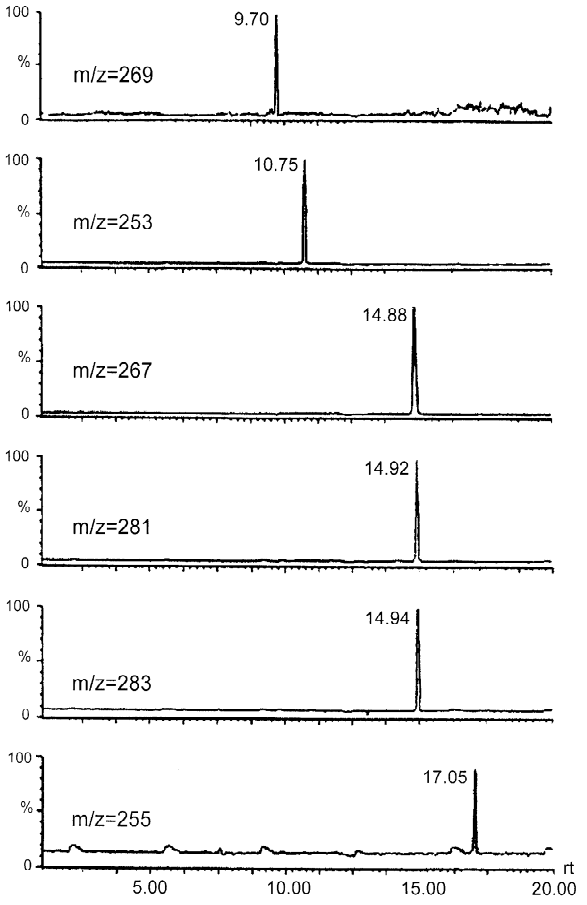

Fig. 9. CE–MS mass chromatograms of isoflavones: *m*/*z* 269, genistein; *m*/*z* 253, daidzein; *m*/*z* 267, formononetin; *m*/*z* 281, pseudobaptigenin; *m*/*z* 283, biochanin A; *m*/*z* 255, isoliquiritigenin. The mixture injected contained 3 fmol of each isoflavone. Modified from Ref. [126] with permission.

3. Non-chromatographic methods

The need for rapid forms of analysis is pervasive. The measurement of phytoestrogens is no exception to this trend. The non-chromatographic methods can be divided into two classes—those that detect and quantitate individual phytoestrogens in mixtures with a high degree of specificity (the immunoassay techniques), and those that exploit specific attributes of each phytoestrogen (deconvolution spectroscopy and MALDI-TOF-MS).

3.1. UV and IR spectroscopy

Spectroscopic methods, such as UV or IR, are not new to the field of analytical chemistry, as they are often used to obtain unique spectra pattern as a support for characterization of purified organic compounds or simple mixtures. However, spectroscopic techniques are less selective without chromatographic separation, especially when applied to complex samples. However, when combined with multivariate data analysis to deconvolute the observed data, they can provide a gross estimation of phenolic composition for characterization of wines, foods and other natural products. UV and IR, near-infrared (NIR) and mid-infrared (MIR) spectroscopy have been applied in the rough analysis of polyphenolic

compounds in wines [128] and soy sauce [129] for the purpose of classification.

Among IR spectroscopic methods, MIR absorption bands are generally well resolved and can be related to defined vibrational transition, while NIR spectra reflect overtones and combination bands of fundamental transition. Edelmann et al. recently reported the use of MIR spectroscopy combined with multivariate data analysis for the discrimination of Austrian red wines [130]. Both authentic phenols and the wine phenolic extracts were investigated. Analysis of untreated wines was difficult, due to the high concentrations of carbohydrates and organic acids in red wines. Both sugars and organic acids feature strong MIR absorption bands between 900 to 1500 cm^{-1}, the region used to characterize phenolic compounds (900–1680 cm^{-1}) in wines. Sample clean up, such as with C_{18} SPE, were necessary for this application. This study demonstrated a method to avoid the time-consuming separation and analysis of single compounds and to take a significant spectrum of the whole phenolic fingerprint instead.

3.2. MALDI-TOF-MS

MALDI-TOF-MS was first introduced by Karas et al. [131] for the analysis of non-volatile and large molecules. In this technique, samples are co-crystallized with a matrix, usually an organic acid, which absorbs energy from the laser pulses and allows a soft desorption ionization of the sample. The sample ions are then analyzed by a TOF mass analyzer. MALDI-TOF-MS has advantages over other methods, including speed of analysis, sensitivity, good tolerance toward contaminants, and the ability to analyze complex samples [132]. Although MALDI-TOF-MS is well known as powerful tool for analysis of a wide range of biomolecules, such as peptides and proteins, its potential in food analysis also have been explored recently [133]. Applications of MALDI-TOF-MS on analyses of anthocyanins and flavonols in red wine and foods have been reported [134–136]. Wang and Spornes [137] demonstrated the first example of using MALDI-TOF-MS to identify isoflavones in soy samples. 2′,4′,6′-Trihydroxy-acetophenone and 2,5-dihydroxybenzoic acid (DHB) were good MALDI matrices for isoflavones. DHB worked well for sample extracts with better spot-to-spot repeatability. In this study, isoflavones exhibited only fragmentation corresponding to loss of their carbohydrate residues. As shown in Fig. 10, daidzin and genistin underwent fragmentation and produced $[M-162+H]^+$ ions at m/z 255 and 271, respectively, corresponding to their agylcones. MALDI spectra of 6″-*O*-malonyl-β-glucoside and 6″-*O*-acetyl-β-glucoside conjugates also contained their corresponding aglycones fragments due to cleavage of the glucosidic group. These fragment ions provided useful information for structural elucidation. The recent introduction of MALDI-Q-TOF and MALDI-TOF-TOF mass spectrometers will enable MS–MS experiments to be carried out, thereby allowing for the structural information to be obtained. This work demonstrates that MALDI-TOF-MS can produce isoflavone profiles of biological samples and serve as a powerful tool to identify and study processing-induced changes of isoflavones in soy products. The one limitation of the MALDI-based approach at this time is that it is not a quantitative technique.

3.3. Immunoassays

Development and application of immunoassay methods offers advantages of specificity, a high rate of sample throughput, and comparatively low cost. Particularly important is the potential high sensitivity. Application of immunoassays to human serum and tissues, where sample volumes are necessarily small, allows study of phytoestrogen absorption and metabolism, and can result in improved assessments of implications for health. Development of structurally specific derivatives of phytoestrogens as haptens is important to raise suitable antibodies for the assays. Based on the nature of detection, three types of immunoassays have been developed for analysis of phytoestrogens, radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and time-resolved fluoroimmunoassay (TR-FIA).

3.3.1. Radioimmunoassay

RIA analysis of formononetin in blood plasma and ruminant fluid of wethers fed on red clover was reported by Wang et al. [138]. The 7-*O*-carboxymethyl ether (CME) of formononetin was synthesized as the hapten and used to raise antisera, as well

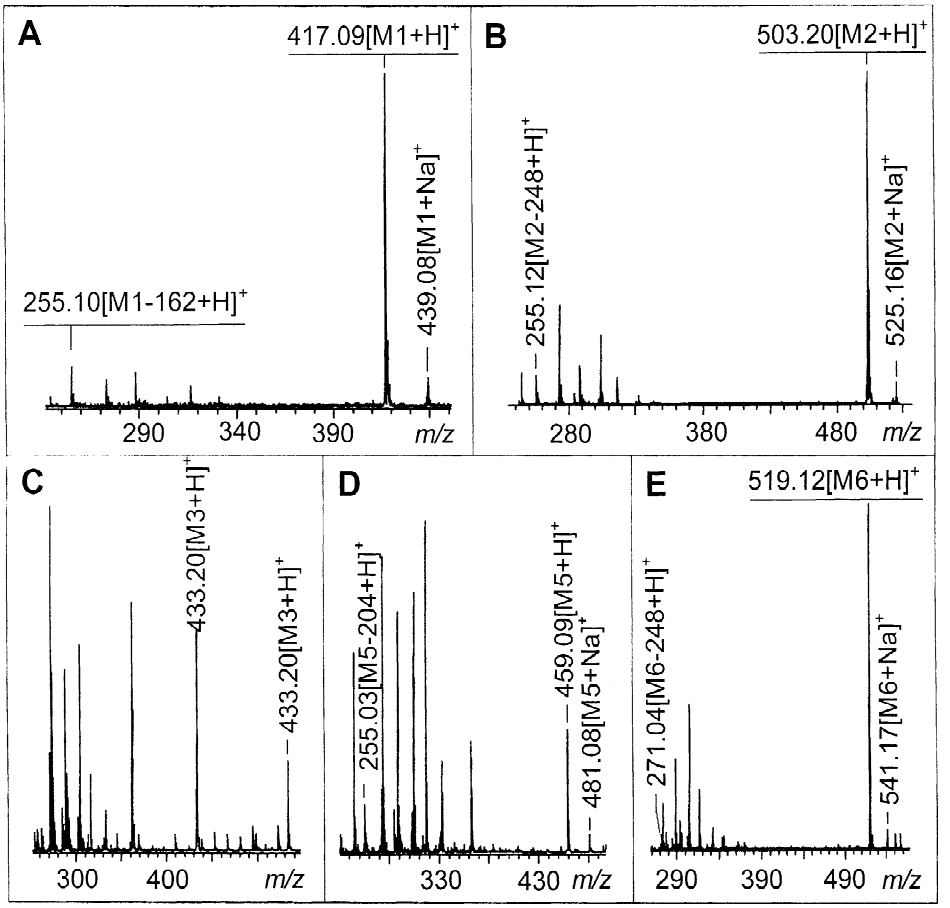

Fig. 10. MALDI-TOF-MS positive ion spectra of isoflavones (from soy flour) after HPLC separation using DHB: (A) peak 1, daidzin; (B) peak 2, 6″-*O*-malonyldaidzin; (C) peak 3, genistin, and 6″-*O*-malonylglycitin; (D) peak 4, 6″-*O*-acetyldaidzin; (E) peak 5, 6″-*O*-malonylgenistin. From Ref. [137] with permission.

as to prepare a [^{3}H]leucine derivative of formononetin as the radioligand. The same method was later applied to determine formononetin in murine plasma and mammary glandular tissue [138]. Antisera showed little or no cross-reaction with structurally related compounds tested, such as genistein, daidzein, and other phytoestrogens. The optimal working range of the RIA was reported as 0.4–40 ng/assay for formononetin standards in physiological saline. However, the sensitivity of this assay was reported as 4 ng/ml (15 n*M*) in plasma samples and 50 ng/g tissue (200 pmol/g or 0.05 ppm) in mammary tissue.

Another RIA for identification of daidzein was reported by Lapcik et al. using antibodies against daidzein-4′-CME-bovine serum albumin (BSA) and ^{125}I-labeled tracer [140]. The sensitivity of this assay was 0.4 pg (1.5 fmol)/tube. The antibodies raised from daidzein-4′-hapten had a 50% cross-reactivity with formononetin. Also, direct RIA values reflected the sum of the free daidzein and its glucuronides and sulfates, due to cross-reaction in this assay. RIA of free genistein in human serum was later established based on polyclonal antibodies against genistein-4′-*O*-(CME)-BSA and genistein-7-*O*-(CME)-BSA conjugates [141]. The sensitivities were reported as 4.4 and 10.4 fmol (1.2 and 2.8 pg/tube), respectively. As the authors anticipated, the 4′-RIA did not distinguish between genistein and its 4′-methoxy derivative, biochanin A, while the 7-RIA system

could not distinguish between genistein and genistin. Since the cross reaction from other structurally related compounds caused overestimation in direct RIAs of isoflavones, these RIA systems were later used to measure isoflavones in beer and plant extracts after HPLC separation [142,143].

3.3.2. Enzyme-linked immunosorbent assay

Kohen et al. [144] described a non-isotopic immunoassay for assessing human exposure to genistein. This method was a competitive enzyme-linked immunoassay using horseradish peroxidase (HRP)-conjugated genistein as tracer and a monoclonal antibody to genistein through the 6-position of genistein. Quantitation of genistein was based on the wall-bound genistein-HRP activity, reacting with tetramethylbenzidine dihydrochloride and hydrogen peroxide, and measurement of product colorimetrically at 450 nm. The antibody used in this report, prepared with the hapten 6-carboxymethyl genistein, showed strong cross reaction with biochanin A and little reaction with genistin, formononetin and apigenin. The sensitivity of this method was reported as 0.5 ng (2 pmol) of genistein per well or 0.5 ng per 10 μl (i.e., 200 n*M*) of urine or plasma samples, which was about 100 times less than the RIA method [141].

3.3.3. Time-resolved fluoroimmunoassay

TR-FIA has been described as a powerful tool for measuring unconjugated phytoestrogens in plasma and tissue samples. This technique which combines the advantages of reagent stability and lack of radiation over RIA, also provides a 10- to 100-fold increase in sensitivity and assay range in comparison with conventional ELISA and FIA methods. This competitive RT-FIA is based on a dissociation-enhanced lanthanide fluoroimmunoassay system, where a nonfluorescent chelate is employed to bind europium to the analytes. After the bioaffinity reaction is completed, the europium ions are dissociated from the chelates by means of an enhancement solution in which lanthanide ions form highly fluorescent complexes with components of the enhancement solution. The enhanced fluorescence is measured at fixed time after excitation of fluorophore. By this time, the background fluorescence has died away. The advantages of this technique, such as high sensitivity, lower background interference, and wide dynamic range, have been reviewed [145].

Adlercreutz et al. [146] developed the first measurement method of phytoestrogen, enterolactone, in plasma by TR-FIA. Similar to all immunoassays, 5′-*O*-carboxymethoxy enterodiol was synthesized and used as a haptene with BSA for preparation of antisera. The 5′-*O*-carboxymethoxy enterolactone was further labeled with europium as a tracer. No antisera cross-activity with lignans, isoflavones, or flavonoids was detected. The sensitivity was reported as 2.1 pg/20 μl (0.4 n*M*), while the working range of this assay varied from 8.9 to 3218 pg/20 μl, corresponding to plasma levels of 1.5–540 n*M*. Analysis of a batch of 100 samples could be completed in 4 h by TR-FIA. Measurements of plasma daidzein [147,148] and genistein [148] were later developed using 4′-*O*-carboxymethoxy derivatives as haptens. Antisera cross reactivities with structurally related compounds were also observed in this report, similar to other immunoassays described before. Finally, the TR-FIA method was used to analyze three phytoestrogens, genistein, daidzein, and enterolactone, in human urine [149,150]. This method has sufficient sensitivity to measure the phytoestrogens at concentrations even below 5 n*M*. Several further applications of these RT-FIA methods also have been reported [151–155].

An essential part of immunoassay is to develop a properly substituted derivative of the target phytoestrogen as a hapten to prepare antisera with good selectivity. Generally the same derivative is also used in preparing tracer for detection. Other than the haptens previously described in this review, a number of synthetic derivatives of phytoestrogens with different spacer arms at various positions also have been described for development of immunoassays [156–159].

4. Conclusion

There is a variety of analytical methods in use for phytoestrogens, most offering sensitivity in the low nmol/l range. A summary of the detection sensitivity of analytical methods for phytoestrogens is listed in Table 3. Generally speaking, if the investigator is working on food samples containing phytoestrogen

Table 3
Reported sensitivities for phytoestrogen analyses

Technique	Limits of detection[b]				Ref.
	pmol	n*M*	ng/ml	ppm[a]	
GC–MS					
Lignans and isoflavones (plasma)		**0.1**			[20]
Lignans and isoflavones (feces)		**1–4**[c]			[32]
8-Prenylnaringenin			5		[31]
Isoflavones, coumestrol and lignans (foods)				**0.02**	[36]
HPLC–UV					
Isoflavones	7.4	370	**100**	**2**	[51]
HPLC–DAD					
Daidzein	0.11	**5.5**	1.3		[45]
Genistein	0.18	**8.8**	2.4		
Formononetin	0.15	**7.3**	2.0		
Biochanin A	0.26	**13.0**	3.7		
Daidzein	1.1	**54.3**	13.8		[48]
Genistein	0.5	**26.6**	9.9		
Equol	3.3	**164.2**	39.7		
O-Desmethylangolensin	1.0	**50.2**	13.0		
Coumestrol	1.3	**67.4**	18.1		
HPLC–fluorescence					
Native					
Coumestrol				**0.5**	[51]
Post-column reaction					
Quercetin	10	0.5	**0.15**		[54]
Kaempferol	4	0.2	**0.05**		
HPLC–ED					
Genistein	**0.2**	37	10		[59]
Genistein	**0.02**	0.19	0.05		[60]
Daidzein	**0.04**	0.39	0.10		
Coumestrol	**0.06**	0.56	0.15		
Daidzein	0.32	**15.8**	4.0		[48,61]
Genistein	0.28	**13.9**	3.8		
Equol	0.59	**29.7**	7.2		
O-Desmethylangolensin	1.70	**85.2**	21.8		
Coumestrol	0.12	**4.9**	**1.3**		[65]
Daidzein	0.13	**5.2**	**1.3**		
Daidzin	0.04	**1.6**	**0.7**		
Equol	0.14	**5.5**	**1.3**		
Enterodiol	0.11	**4.4**	**1.3**		
Enterolactone	0.12	**4.9**	**1.3**		
Genistein	0.18	**7.3**	**2.0**		
Daidzin	**0.07**	7.3	3.1		[66]
Genistin	**0.09**	9.3	4.0		
Secoisolariciresinol	**0.01**	0.9	0.3		
Dihydrodaidzein	**0.05**	4.9	1.3		
Daidzein	**0.05**	4.6	1.2		
Enterodiol	**0.02**	1.9	0.6		
Dihydrogenistein	**0.06**	5.5	1.5		
Matairesinol	**0.02**	1.8	0.7		
Equol	**0.03**	2.5	0.0		
Enterolactone	**0.02**	2.1	0.6		
Genistein	**0.09**	9.3	2.5		
O-Desmethylangolensin	**0.03**	3.3	0.9		
Anhydrosecoisolariciresinol	**0.02**	1.6	0.5		

Table 3. Continued

Technique	Limits of detection[b]				Ref.
	pmol	n*M*	ng/ml	ppm[a]	
HPLC–MS					
Daidzein (in urine samples)			**0.025**[d]		[75]
Genistein			**0.0027**		
Daidzein (in flour and food samples)				**0.2**	[77]
Genistein				**0.7**	
Isoflavones	0.3	**15**	3.9		[73]
CE–UV(DAD)					
Genistein	0.015	1480	**400**		[113]
Daidzin	0.043	4255	**1770**		[114]
Biochanin A	0.048	4800	**1370**		
Puerarin	0.040	3990	**1660**		
Daidzein	0.048	4800	**1220**		
Geniatein	0.049	4900	**1330**		
Daidzein	0.055	5500	**1400**		[117]
CE–FL					
Isoflavones (daidzein, formononetin, etc.)	0.001	**100–400**	27–108		[118]
CE–ED					
Daidzein	0.0010	**190**	748		[121]
Quercetin	0.0011	**225**	745		
Daidzein	0.0012	**241**	948		[122]
Puerarin	0.0017	**344**	826		
CE–MS (30 nl)					
Isoflavones (daidzein, formononetin, etc.)	**0.0001**	**6**	22.2		[126]
UV and IR spectra	NA	NA	NA	NA	
MALDI					
Isoflavones (genistin, daidzin, etc.)	NA	NA	NA	NA	[137]
Immunoassay					
RIA (^{3}H)					
Formononetin	**0.7**	1.4	0.38		[138]
Formononetin (in plasma or tissue)			**4**	**0.05**	[139]
RIA (^{125}I)					
Daidzein	**0.0016**	0.016	0.004		[140]
Genistein	**0.0044**	0.044	0.012		[141]
Daidzein	0.008	**0.08**	0.002		[142]
Genistein	0.015	**0.15**	0.004		
ELISA					
Genistein	**1.85**	**185**			[144]
TR-FIA					
Enterolactone	**0.007**	**0.35**	0.1		[146,149]
Daidzein (in urine)	**0.020**	**2.0**	0.5		[147]
Daidzin	**0.006**	**0.6**	0.25		
Genistein (in plasma)	**0.011**	**0.55**	0.015		[148,149]
Daidzein	**0.007**	**0.35**	0.089		

[a] ppm=μg/g or mg/kg.

[b] The **bold** numbers represent the limits of detection reported in the original references. A wide range of units of detection limit are converted based on the injection volumes reported. For all CE analyses, the injection volumes are estimated based on the volumes in 0.5 cm of capillaries used for separation.

[c] The limits of detection represent 1–4 nmol in feces collected in 24 h period.

[d] The limits of detection reported in this reference were the lowest detectable levels of isoflavones in 3 ml of urine samples analyzed by solid-phase microextraction and HPLC with ion-trap MS. It does not represent the lowest level for quantitative analysis.

concentrations that are greater than 50 ppm (this is largely restricted to soybean or red clover products), HPLC with DAD–UV detection is the method of choice since it is equally suited to the quantitative measurement of both aglycones and glycosides and requires minimal sample preparation. If the importance of quantitation is low, then MALDI-TOF-MS is an exciting new development because its high speed (100 samples/h) and sensitivity.

For food and/or biological samples that have phytoestrogen concentrations less than 50 ppm (food or tissue) or 200 n*M*, or which are less than 1 g or 1 ml, HPLC–UV is not adequate. Detection using fluorescence may help in certain cases. For measurement of single phytoestrogens in large numbers of samples (e.g., for confirming compliance in a clinical trial or estimating exposure in an epidemiological study), immunoassay methods are ideal, with TR-FIA the method of choice. For well-described matrices where there are many known phytoestrogens present, LC–ED is suitable. For studies where the phytoestrogens in the sample are not known a priori, methods based on mass spectrometry are recommended. GC–MS, although very laborious, offers high sensitivity and, with isotope dilution, high precision. LC–MS requires very little sample work up and provides the opportunity to determine the chemistry of the phytoestrogens and their metabolites, even in the conjugated form. If miniaturized in a microfluidic system, they can offer sensitivity down to the low fmol range. A similar sensitivity can be achieved with HPLC in combination with electrochemical detectors although this method has a limited ability to discriminate between phytoestrogens and therefore cannot be used to predict phytoestrogen structure.

Capillary electrophoresis is a powerful separation method for phytoestrogens, particularly when combined with electrochemical detection and mass spectrometry. However, CE–MS remains a challenge to setup and may be restricted to laboratories with considerable technical capability.

5. Nomenclature

APCI	Atmospheric pressure chemical ionization
BSA	Bovine serum albumin
CE	Capillary electrophoresis
CID	Collision-induced dissociation
CME	Carboxymethyl ether
CZE	Capillary zone electrophoresis
DAD	Diode array detection
DHB	2,5-Dihydroxybenzoic acid
DMSO	Dimethyl sulfoxide
ED	Electrochemical detection
EI	Electron impact ionization
ESI	Electrospray ionization
ELISA	Enzyme-linked immunosorbent assay
GC	Gas chromatography
GC–MS	Gas chromatography–mass spectrometry
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
ID–GC–MS	Isotope dilution GC–MS
IR	Infrared
LC–MS	HPLC–mass spectrometry
LIF	Laser-induced fluorescence
MS	Mass spectrometry
MALDI-TOF-MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MEKC	Micellar electrokinetic chromatography
MRM	Multiple reaction ion monitoring
MIR	Mid-infrared
NIR	Near-infrared
Q-TOF-MS	Hybrid quadrupole orthogonal time-of-flight mass spectrometry
RIA	Radioimmunoassay
SDS	Sodium dodecyl sulfate
SPE	Solid-phase extraction
TMS	Trimethylsilyl ether
TR-FIA	Time-resolved fluoroimmunoassay
UV	Ultraviolet

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