Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 812 (2004) 325–355
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Review

Analytical methods to determine phytoestrogenic compounds

Qingli Wu*, Mingfu Wang, James E. Simon

New Use Agriculture and Natural Plant Products Program, Department of Plant Biology and Pathology, Cook College, Rutgers University, New Brunswick, NJ 08901-8520, USA

Received 5 April 2004; accepted 2 August 2004
Available online 11 September 2004

Abstract

The analytical methods for the determination of phytoestrogenic compounds in edible plants, plant products and biological matrices are reviewed. The detection, qualitative and quantitative methods based on different chromatographic separations of gas chromatography (GC), high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) coupled with various detections by ultraviolet absorption (UV), electrochemical detection (ED), fluorescence detection, mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR), as well as non-chromatographic immunoassay are each extensively examined and compared. An overview on phytoestrogen chemistry, bioactivities and health effects, plant precursors, metabolism and sample preparation is also presented.

Keywords: Review; Phytoestrogen; Analytical method; Isoflavone; Lignan

Contents

1. Introduction 326
   1.1. Chemistry of phytoestrogenic compounds and natural sources 326
   1.2. Metabolism of phytoestrogenic compounds 328
2. Analytical methods 329
   2.1. Gas chromatography–mass spectrometry 329
   2.2. High-performance liquid chromatography-based methodology 332
      2.2.1. Ultraviolet detection 332
      2.2.2. Electrochemical detection 339

Abbreviations: ACN, acetonitrile; ADME, absorption, distribution, metabolism and excretion (new drug discovery); APCI, atmospheric pressure chemical ionization; API, atmospheric pressure ionization; BSA, bovine serum albumin; CE, capillary electrophoresis; CEAD, coulometric electrode array detector; CEC, capillary electrochromatography; CME, carboxymethyl ether; CV, coefficient variation; CZE, capillary zone electrophoresis; DMSO, dimethyl sulfoxide; ED, electrochemical detection; EI, electron ionization; ESI, electrospray ionization; ELISA, enzyme-linked immunosorbent assay; FAB-MS, fast atom bombardment-mass spectrometry; FT-ICR-MS, Fourier-transform ion-cyclotron resonance mass spectrometry; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; HRMS, high resolution mass spectrometry; i.d., inter-diameter; ID-GC–MS, isotope dilution–gas chromatography–mass spectrometry; IS, internal standard; IT-MS, ion trap-mass spectrometry; LC–MS, liquid chromatography–mass spectrometry; LIF, laser-induced fluorescence; LOD, limit of detection; LOQ, limit of quantitation; 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; NACE, nonaqueous capillary electrophoresis; NMR, magnetic resonance spectroscopy; PDA, photodiode array detector; Q-TOF-MS, hybrid quadrupole orthogonal time-of-flight mass spectrometry; RDA, retro-Diels–Alder;RIAradioimmunoassay; RP, reversed phase; R.S.D., relative standard deviation; SDS, sodium dodecyl sulfate; SIM, selected ion monitoring; SPE, solid-phase extraction; SRM, selected reaction monitoring; TFA, trifluoroacidic acid; TIC, total ion chromatogram; TMS, trimethylsilyl ether; TR-FIA, time-resolved fluoroimmunoassay; TSP, thermospray (interface); UV, ultraviolet

* Corresponding author. Tel.: +1 732 932 9711x372; fax: +1 732 932 9441.
E-mail address: qlwu@aesop.rutgers.edu (Q. Wu).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.08.008

2.2.3. Fluorescence detection ... 339
2.2.4. Mass spectrometry ... 340
2.2.5. Nuclear magnetic resonance spectroscopy ... 343
2.3. Capillary electrophoresis-based methodology ... 343
2.3.1. Capillary zone electrophoresis ... 348
2.3.2. Micellar electrokinetic capillary chromatography ... 349
2.3.3. Capillary electrochromatography ... 349
2.4. Immunoassays ... 349
2.4.1. Radioimmunoassay ... 350
2.4.2. Enzyme-linked immunosorbent assays ... 350
2.4.3. Time-resolved fluoroimmunoassay ... 350
2.5. Sample preparation ... 351
3. Conclusions ... 351
Acknowledgments ... 352
References ... 352

1. Introduction

Phytoestrogenic compounds are secondary plant metabolites which have estrogen-like properties and have been associated with lesser incidence of steroid-hormone dependent cancers, e.g. those of the breast, prostate and colon [1–7]. The major classes of phytoestrogenic compounds, which in this review will be termed phytoestrogens, are phenolic natural products of isoflavones and lignans, as well as coumestanes, flavonoids and stilbenes [8–15]. As postmenopausal estrogen treatment is associated with a lower cardiovascular disease risk, but may lead to a higher risk of endometrial cancer, breast cancer and venous thrombosis, consumption of phytoestrogens as a replacement hormone therapy has been a major area of research since these compounds were reported to decrease the risk of cardiovascular disease coupled with a lower risk of several types of cancer [16–20]. Epidemiological studies also revealed that the incidence of breast cancer for Japanese and Chinese women is lower than that for women in the West [7], and the prevailing contribution to this difference has been attributed to the diet. One of the most important staple foods in the Asian diet is soy and soy derived products, which contain abundant isoflavone phytoestrogens including genistein, daidzein, glycitein and their glycoside derivatives. Furthermore, a clinical study also indicated that the concentration of daidzein (one of the major isoflavones in soy) in 24-h urinary excretion from Japanese women was about 10 times higher than that from recent Asian women immigrants to Hawaii, and their breast cancer rates are also greater [1,2]. Rye bread enriched in lignans is another principle phytoestrogen sources. The women in the east of Finland, known for their high consumption of whole-grain rye bread as dietary biomarker, show a lower incidence of breast cancer either than those in Western Europe and USA [7,21]. Phytoestrogens and their metabolites have been detected in various human and animal biological fluids. Based on the structural similarity to the endogenous estrogens, e.g. 17-β-estradiol, phytoestrogens with 2-phenylnaphthalene-type chemical structures have been found to bind to estrogen receptors [4,17,20,22]. Recent investigations also indicated that many of their potential health benefits may be attributable to metabolic properties instead of involving estrogen receptors, such as their influence on enzymes, protein synthesis, cell proliferation, calcium transport, Na^+/K^+ adenosine triphosphatase, angiogenesis, vascular smooth muscle cells, growth factor action, lipid oxidation, and cell differentiation [22–24]. As a consequence, there have been an increasing number of studies on phytoestrogens over the past 20 years covering a wide scientific range of disciples including plant origins, chemistry, bioactivity, preclinical and clinical study. The number of scientific published papers on phytoestrogens rose from 15 in 1980 to nearly 600 in 2002 (Fig. 1). Therefore, the ability to characterize and quantitate the phytoestrogenic precursors in plants and/or plant derived products and their metabolites in biological matrices has become a serious area of research inquiry. As this same time period was associated with a dramatic development of newer analytical techniques, there have been a wide array of assays employed using different chromatographic separations combined with a wide variety of detectors. Wang et al. [8] presented a comprehensive review on phytoestrogen determination though 2001, but they focused primarily on isoflavones, while other reviews on phytoestrogen lignans only gave a brief overview on the analytical methods [4,9,25]. While a large number of analytical methods have been published, in this paper we seek to review the analyses of phytoestrogenic isoflavones from 2001 and extensively examine the analytical methods used for the determination of phytoestrogen lignans in plant, plant derived products and biological matrices. Our goal is therefore to review the literature and present a comparative discussion on the multitude of analytical methods now available and where future work appears to be moving.

1.1. Chemistry of phytoestrogenic compounds and natural sources

Isoflavones as one of the major classes of phytoestrogens are widely distributed in plant kingdom, but accumulate pre-

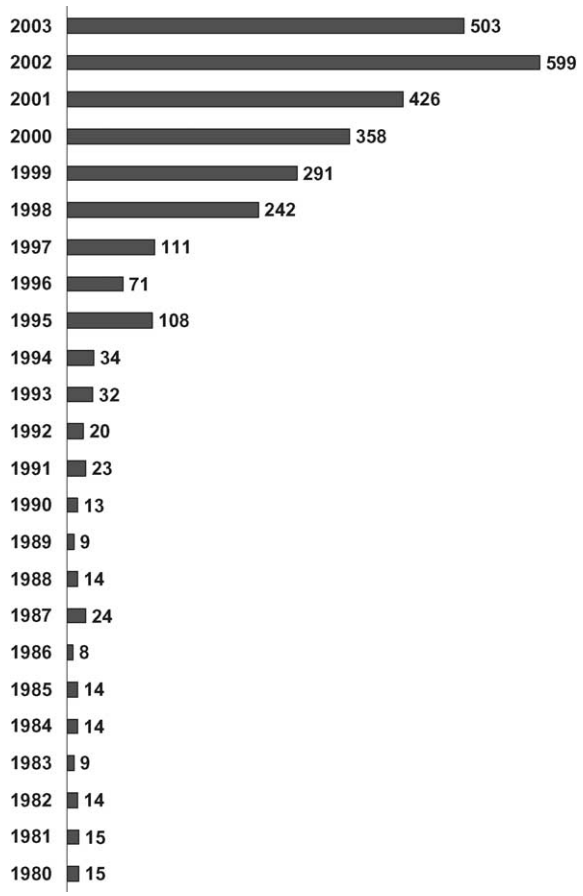
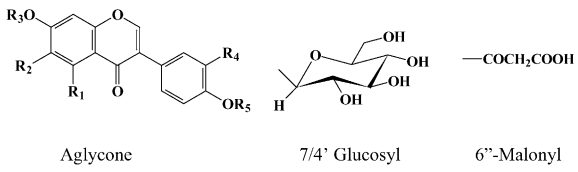


Fig. 1. Number of published papers per year related to phytoestrogens from 1980 (data from SciFinder Scholar, July 2004, search keyword phytoestrogen).

dominantly in plants of the Leguminosae family. The best-known sources of phytoestrogenic isoflavones are soy and soy derived products, which have been a traditional food for eastern Asian populations for thousands years. Daidzein, genistein and their glycoside conjugates represent the major soy isoflavones, along with small amounts of glycitein and its glycoside derivatives. These compounds are also present in significant concentrations in various beans and sprouts [1,7,26–30]. Red clover (*Trifolium pratense*) is a rich source of isoflavones and red clover based products are commercially available as dietary supplements in the US and European markets for women suffering menopausal complaints. In red clover, the majority of isoflavones include formononetin, biochanin A and their glycoside malonates, with smaller concentrations of daidzein, genistein and glycitein derivatives known as the principle phytoestrogens in soy [31–35]. The isoflavone precursors detected in soy and red clover are illustrated in Fig. 2. The glucosyl, occasionally the galactosyl group is generally substituted on 7 or 4′ position of isoflavone aglycone skeleton, and the malonyl group linked to 6″ position of sugar moiety. As isoflavone plant precursors, these compounds are generally present as 7-β-D-glucosides and 6″-*O*-malonylglucosides because these conjugated forms can be utilized to store the less soluble isoflavone aglycones. Other



	R_1	R_2	R_3	R_4	R_5
Daidzein (**1**)	H	H	H	H	H
Genistein (**2**)	OH	H	H	H	H
Glycitein (**3**)	H	OMe	H	H	H
Formononetin (**4**)	H	H	H	H	Me
Biochanin A (**5**)	OH	H	H	H	Me
Pseudobaptigenin (**6**)	H	H	H		OCH_2-
Prunetin (**7**)	OH	H	Me	H	H
Calycosin (**8**)	H	H	H	OH	Me
Irilone (**9**)	OH		OCH_2-	H	H
Pratensein (**10**)	OH	H	H	OH	Me

Fig. 2. Chemical structures of isoflavone phytoestrogenic compounds in both soy and red clover, two primary rich plant sources of isoflavones. Daidzein, genistein and their glycoside derivatives are the major isoflavones in soy, while formononetin, biochanin A and their malonyl derivatives are predominant isoflavones in red clover.

leguminous plants containing isoflavones include *Pueraria* spp. (kudzu) [36,37], *Glycyrrhiza* spp. (licorice) [38], *Astragalus* spp. (milkvetch) [39], *Sophora japonica* (Japanese pagodatree) [40], *Sophora flavescens* (lightyellow sophora) [41], *Baptisia* spp. [42], *Millettia* spp. [43], *Dalbergia* spp. [44], *Lupinus* spp. [45], *Ateleia* spp. [46], *Desmodium uncinatum* [47] and *Derris* spp. [48], together with other species distributed in other taxons, such as *Henriettella fascicularis* (Melastomataceae) [49], *Iris germanica* (Iridaceae) [50] and *Maclura pomifera* (Moraceae) [51].

Phytoestrogenic lignans are also present in a wide variety of plants with many oilseeds, vegetables and fruits known to contain lignan precursors. Flaxseed (*Linum usitatissimum*) is among the richest known source of these compounds, but the consumption of flaxseed products in human diets is relatively low. As a consequence, it is the cereal and whole-grain products, particularly rye and barley that provide the most important dietary source of lignan precursors [9,25,52,53]. Many oilseeds, vegetables and fruits have been also found to contain lignan precursors. Previous reports have summarized their availability and contents in different plants and food sources [9,17,22,54]. Lignan, first named by Harworth in 1941 [55], is a dimeric natural product derived by the combination of two phenylpropanoid C6–C3 units at β carbon atoms. Enterodiol (**11**) and enterolactone (**12**) are the two most common mammalian lignans and have been described as the major lignans present in serum, urine, bile and seminal fluids of humans and animals, as well as 7′-hydroxyentrolactone (**13**) and enterofuran (**14**) identified more recently in human urine [56–61]. In humans and animals, two of the most common lignan precursors known to form enterodiol (**11**) and enterolactone (**12**) upon bacterial fermentation are secoisolariciresinol (**16**) and matairesinol (**15**), respectively [4,9,25,62,63]. Enterolactone (**12**) can be also converted by enterodiol (**11**). Glycoside derivatives of matairesinol (**15**)

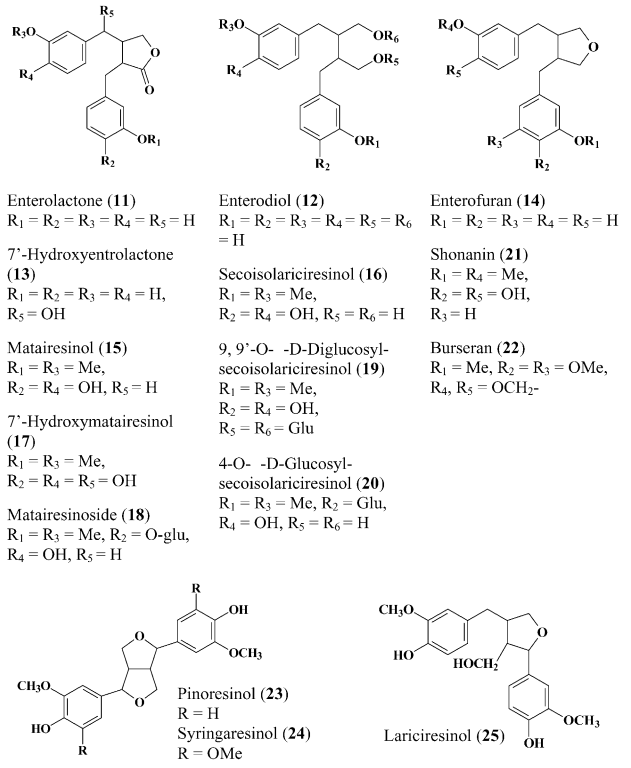


Fig. 3. Chemical structures of mammalian lignans (**11**–**14**) and examples of common plant lignan precursors (**15**–**25**).

and secoisolariciresinol (**16**), such as matairesinoside (**18**), 9, 9′-*O*-β-D-diglucosylsecoisolariciresinol (**19**) and 4-*O*-β-D-glucosylsecoisolariciresinol (**20**) are the major components of flaxseed and possibly the lignan precursors of enterodiol (**11**) and enterolactone (**12**). These glycoside derivatives are easily hydrolyzed and metabolized by colonic microflora to the mammalian lignans [9,62,64,65]. In addition, 7′-hydroxymatairesinol (**17**), pinoresinol (**23**), syringaresinol (**24**) and lariciresinol (**25**) have also been recently identified as precursors of mammalian lignans, and the last three compounds have been found in cereals, particularly in whole-grain rye derived products [21,25,66]. The dibenzylteterahydrofuran lignans shonanin (**21**) and burseran (**22**) found in flax have been also considered as lignan precursors [60,67]. The structures of mammalian lignans (**11**–**14**) and examples of common plant precursors (**15**–**25**) are illustrated in Fig. 3. Other phenolic phytoestrogens include flavanoids, such as 8-prenylnaringenin (**30**) and related flavonoids in hops (*Humulus lupulus*) and beer [68,69], coumestrans of coumestrol (**29**) in alfalfa (*Medicago sativa*) [12,13,70], and *trans*-resveratral (**31**) enriched in grape and grape wine [14,15].

1.2. Metabolism of phytoestrogenic compounds

Glycoside derivatives of isoflavone and lignan, the two major forms of phytoestrogen precursors in plants are converted to hormone-like aglycones by intestinal bacteria (parent compounds) in both humans and animals. The parent

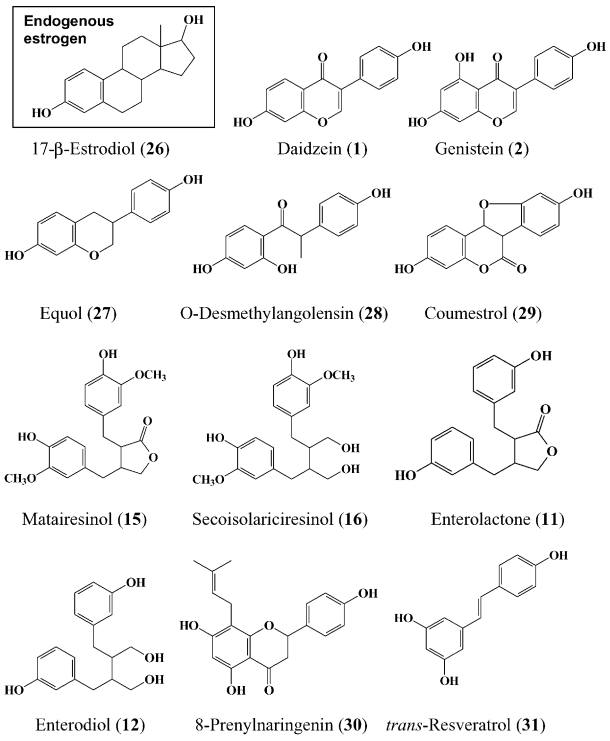


Fig. 4. Chemical structures of endogenous estrogen 17-β-estrodiol, common naturally occurring phytoestrogenic compounds and important metabolites showing a marked similarity to estrogen. Equol (**27**) and *O*-desmethylangolensin (**28**) are important metabolites of isoflavone precursors. The mammalian lignans enterolactone (**11**) and enterodiol (**12**) are formed from lignan precursors by internal bacteria.

compounds, such as daidzein (**1**), genistein (**2**), matairesinol (**15**) and secoisolariciresinol (**16**) are then directly absorbed or further metabolized into a wide range of metabolites [22,31,63,64]. The chemical structures of the common naturally occurring phytoestrogenic compounds and their important metabolites show a striking similarity to endogenous estrogen of 17-β-estrodiol (Fig. 4).

Soy and red clover are the richest natural sources of isoflavone phytoestrogens. In these plants, isoflavones are predominantly present as the glycoside and glycoside malonate conjugates, such as 7-*O*-β-D-glucoside and 6″-*O*-malonylglucose of daidzein and genistein in soy, and 7-*O*-β-D-glucoside and 6″-*O*-malonylglucose of formononetin and biochanin A in red clover [31]. These conjugates are of biological interest in plants and can be utilized to store the less soluble isoflavone aglycones. Upon ingestion, they are first hydrolyzed by intestinal bacteria to release aglycones, which are then metabolized further [71,72]. Formononetin (**3**) and biochanin A (**4**), 4′-methyl ether derivatives, are demethylated to daidzein (**1**) and genistein (**2**) in humans and animals, respectively [1,2]. Daidzein (**1**) is then reduced to equol (**27**) and *O*-desmethylangolensin (**28**), while genistein (**2**) to 6-hydroxy-*O*-desmethylangolensin, 2-(4-hydroxyphenyl)propanoic acid and tri-hydroxybenzene [4,20,22,26,73–75]. The major biotransformations in metabolism of isoflavone phytoestrogens in humans and an-

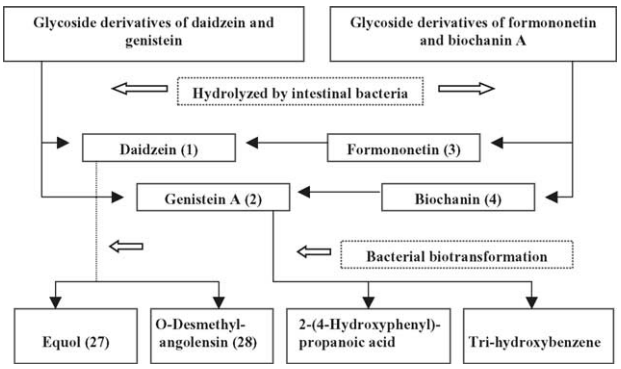


Fig. 5. Metabolic pathways of the major isoflavone phytoestrogens. Equol (**27**), *O*-desmethylangolensin (**28**), 2-(4-hydroxyphenyl)propanoic acid and tri-hydroxybenzene are among the most important metabolites.

imals are illustrated in Fig. 5. The isoflavone aglycones and their metabolites are absorbed in the intestine, where they are mostly conjugated into glucuronides and/or sulphates prior to release into the blood, and transported to the liver. Few exit as free aglycones [26,74]. Apart from reductive and conjugative metabolism, the oxidative biotransformation of isoflavones has been also reported in rats and humans [76–78].

Enterolactone (**11**) and enterodiol (**12**) as the most important mammalian lignans were first found in human urine [79,80], as well as newly identified enterofuran (**14**) and 7′-hydroxymatairesinol (**17**) [60]. Glycoside derivatives of matairesinol (**15**) and secoisolariciresinol (**16**), such as matairesinoside (**18**), 9,9′-*O*-β-D-diglucosylsecoisolariciresinol (**19**) and 4-*O*-β-D-glucosylsecoisolariciresinol (**20**) are the major plant lignans detected in flaxseed. In humans and animals, they are first hydrolyzed to their aglygones matairesinol (**15**) and secoisolariciresinol (**16**) as two primary plant precursors of mammalian lignans by the β-glycosidase, which is widespread in intestinal microorganisms. Matairesinol (**15**) and secoisolariciresinol (**16**) are then converted to enterolactone (**11**) and enterodiol (**12**) by the activity of the gut microflora, respectively, and enterodiol (**12**) could be subsequently converted to enterolactone (**11**) [9,62,64,65]. The plant lignans pinoresinol (**23**), syringaresinol (**24**) and lariciresinol (**25**) are recently found to be new lignan precursors for mammalian lignans enterolactone (**11**) and enterodiol (**12**) after human fecal incubation [21,66]. Until recently, enterolactone (**11**) and enterodiol (**12**) as the most common mammalian lignans have been detected in a variety of biological matrices of human plasma, prostate fluids, urine and fecal. Small amount of unchanged parent lignans, such as matairesinol (**15**), secoisolariciresinol (**16**) and lariciresinol (**25**) have been also detected in human urine [25,56,81]. The metabolic pathway of main lignan phytoestrogens is illustrated in Fig. 6.

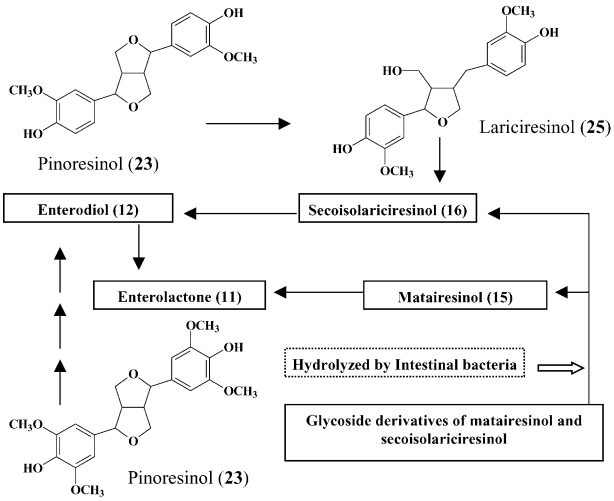


Fig. 6. Metabolic pathways of the lignan type phytoestrogens. Enterolactone (**11**) and enterodiol (**12**) are the major mammalian lignans.

2. Analytical methods

In the last two decades, many analytical methods for detection and quantitation of phytoestrogens and their metabolites in plants, plant derived products and biological matrices have been reported. When classified upon separation methodology, the analytical methods can be categorized into chromatographic and non-chromatographic. Included within the chromatographic methods are gas chromatography (GC), high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) coupled with various detections by ultraviolet absorption (UV), electrochemical detection (ED), fluorescence detection, mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR). Included within the non-chromatographic methods are matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF-MS) and immunoassay.

2.1. Gas chromatography–mass spectrometry

Prior to the application of LC–MS, GC–MS had been the most commonly used technique for the analysis of phytoestrogenic compounds and their metabolites in biological fluid due to its marked potential of high resolution, selectivity and sensitivity. Even now, for routine analysis of volatile organics, high-resolution GC directly combined with now the wide range of low-cost bench-top MS instruments remain a most attractive technique. In 1959, Gohlke first described the direct introduction of GC effluent into a time-of-flight (TOF) mass spectrometer equipped with an oscilloscope [82], and only a few years latter, analyses of different volatile organic compounds using this new technique were reported [83,84]. Over time, direct coupling of a GC unit with various capillary columns to different MS analyzers, i.e. magnetic sectors, linear quadrupoles, ion traps and TOF analyzers have been successfully employed, and several ionization techniques including electron ionization (EI) and chemical ionization (CI) have also been employed.

The choice of MS analyzer to employ directly relates to user's need for mass range, mass resolution, sensitivity, spec-

tral collection speed, and cost of the instrument. TOF-MS as a non-scanning mass analyzer provides very fast acquisition rates, high mass range, and/or high MS resolution, but this device is more expensive than that of the lower-resolution quadrupole or ion trap instruments. Furthermore, the feature of high mass range for TOF-MS is less necessary when used in combination with GC, which is predominately applied for analysis of low mass analytes. Magnetic sector MS instruments can offer high sensitivity, a relatively wide mass range, quite high scanning speed, and/or high mass resolution, but the significant higher cost and space needs for the instrument limit its application. Alternatively, the ion trap MS detector (IT-MSD) permits the use with the additional benefits of enhanced selectivity through multiple stage MS (MS^n) with little or no additional capital expense in comparison with quadrupole MS instruments. Quadrupole MS has the advantaged of relatively lower cost, compactness, reliability, electronically controlled resolution and simplicity of operation. Additionally, for compound identification, this unit is favored as most of the MS library spectra were generated by quadrupole MS detectors. The majority of GC–MS applications now utilize bench-top instruments with linear quadrupoles and electron ionization, and generally under the operation mode of full scan and selected monitoring (SIM). These different MS analyzers including their design and feathers will be discussed further in the following LC–MS section.

All phytoestrogen precursors as they exit in the plant and their metabolites in biological matrices contain polar hydroxyl and/or carboxyl groups. Derivatization is therefore needed to increase the volatility of each analyte for their analysis by GC–MS, resulting in superior chromatographic properties, such as improved peak shape. Moreover, the formation of derivatives may enhance the thermal stability and the ionization efficiency or selectivity of MS, which would be helpful for the identification and quantitation of target compounds. The common reagents include *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA-TMCS) and produce the derivatives of trimethylsilyl ether (TMS). Samples may also be silylated with pyridine–hexamethyldisilazane–trimethylchlorosilane (Py–HMDS–TMCS) (9:3:1, v/v/v) to form TMS derivatives [85]. GC–MS analysis generally involves a multi-step sample preparation, such as organic solvent extraction and solid-phase extraction (SPE, C-18, shephadex-LH20 or ion-exchange chromatography) for sample pre-purification; hydrolysis of conjugates (β-glucuronidase and/or sultfatase) and derivatization to increase compound volatility. The potential and unknown losses during this processing need to be understood. The requirement of an internal standard (IS) is critical to correct for such losses. These standards are usually achieved by compounds carrying stable isotopic labels such as deuterated (^{2}H) or carbon-13 (^{13}C) labeled forms of the corresponding phytoestrogens and their metabolites, or chemical analogous with similar chemical structure and properties that are not naturally present in the sample to be studied [86].

Adlercreutz and coworkers [81,85,87–94], over the last two decades, have made extensive investigations on phytoestrogen pharmacokinetics using GC–MS. Adlercreutz et al. [91] first presented a method based on isotope dilution GC–MS (ID-GC–MS) for the quantitation of phytoestrogenic lignans and isoflavones, and their metabolites in biological matrices under the operation mode of selected ion monitoring (SIM). This method or a slightly modified method has become a routine assay for determination of phytoestrogens and their metabolites in both plant/plant derived samples [75,93,95,96] and biological samples [61,66,97–106]. Apart from the isoflavone metabolites, dihydrodaidzein, dihydrogenistein, equol, *cis*-4-OH-equol, *O*-desmethylangolensin and 6′-OH-*O*-desmethylangolensin, and mammalian lignans enterolactone and enterodiol previously reported in biological samples [85,87,88], Heinonen et al. [94] recently reported the identification of several other new isoflavone metabolites from human subjects by GC–MS. In their study, the metabolism of phytoestrogenic isoflavones daidzein, genistein and glycitein was examined in human urine. The sample preparation procedure and GC–MS conditions used for this study were modified as described earlier [85]. Urine samples were first extracted with C-18 cartridges, then hydrolyzed with β-glucuronidase/sulphatase from *Helix pomatia*. Hydrolyzed matrices were then extracted with diethyl ether and treated again with C-18 cartridges. After pre-purification, the samples were silylated with QSM (Py–HMDS–TMCS, 9:3:1, v/v/v). Isolation and characterization of the urinary metabolites were carried out using gas chromatography/electron ionization mass spectrometry (GC/EI-MS), and several new metabolites were identified by the interpretation of mass spectra (Fig. 7). These new metabolites were 5′-OMe-*O*-desmethylangolensin, 5′-OH-*O*-desmethylangolensin, 3″-OH-*O*-desmethylangolensin, 3′-OMe-equol, 6-OMe-equol, 3′,4′,7-trihydroxyisoflavan, 3′,4′,7-trihydroxyisoflavanone, 4′,7,8-trihydroxyisoflavanone, 4′,6,7-trihydroxyisoflavanone, 3′,4′,7-trihydroxyisoflavone, 4′,7,8-trihydroxyisoflavone, 3′,4′,5,7-tetrahydroxyisoflavanone and 4′,6,7-trihydroxyisoflavanone, and were found in additional to other previously reported metabolites.

Using GC coupling with ion trap MS detector (GC/IT-MSD), Sicilia et al. [67] presented a comparative study on two types of flaxseed (*L. usitatissimum* L. and *L. flavum*) known to be the richest lignan sources and pumpkin seeds (*Cucurbita pepo* L.) regarding their chemical profile of lignans under enzymatic and acidic hydrolysis, respectively. GC–MS analysis of the plant extracts revealed secoisolariciresinol as the major lignan compound, and traces of lariciresinol were also detected for the first time in both flaxseeds and pumpkin seeds. Artificial products and loss of original lignans under acidic conditions were observed, and stereochemistry of flaxseed lignans were analyzed by chiral HPLC showing that secoisolariciresinol, matairesinol, and lariciresinol consisted predominantly of one enantiomorph. Finely grounded plant

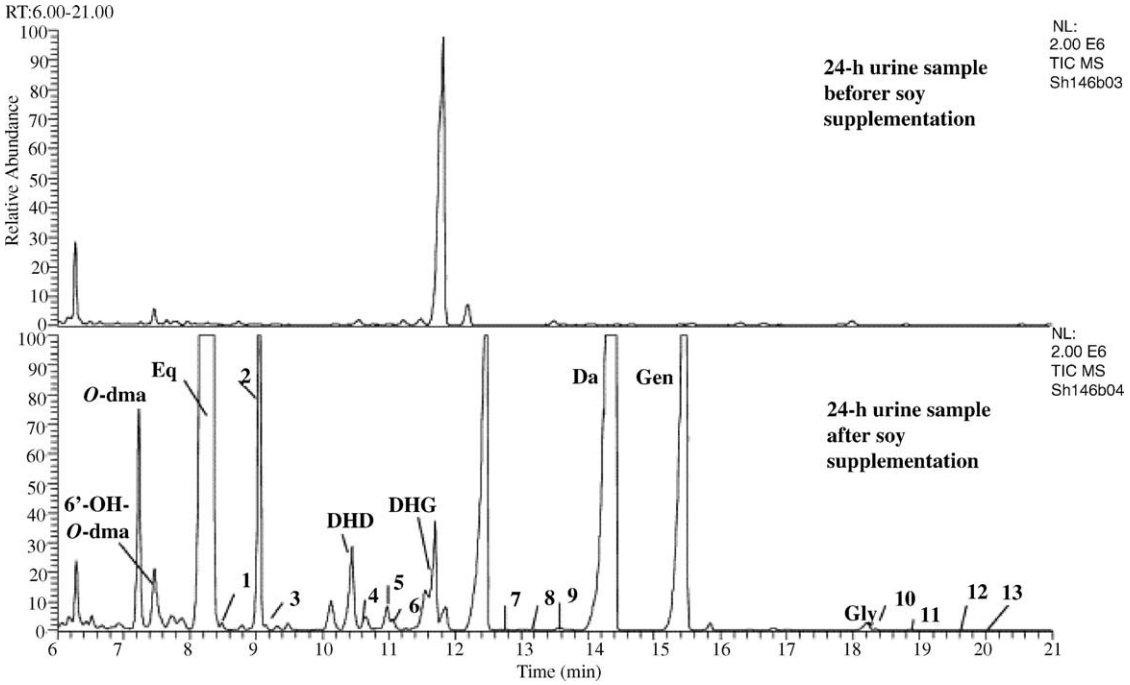


Fig. 7. Total ion chromatograms (TIC) of urine sample extracts of so-called equol-producers prior to and following soy supplementation. The metabolites that were identified are: 5′-OMe-*O*-desmethylangolensin (**1**); 5′-OH-*O*-desmethylangolensin (**2**); 3″-OH-*O*-desmethylangolensin (**3**); 3′-OMe-equol (**4**); 6-OMe-equol (**5**); 3′,4′,7-trihydroxyisoflavan (**6**); 3′,4′,7-trihydroxyisoflavanone (**7**); 4′,7,8-trihydroxyisoflavanone (**8**); 4′,6,7-trihydroxyisoflavanone (**9**); 3′,4′,7-trihydroxyisoflavone (**10**); 4′,7,8-trihydroxyisoflavone (**11**); 3′,4′,5,7-tetrahydroxyisoflavanone (**12**); and 4′,6,7-trihydroxyisoflavanone (**13**). Da, daidzein; Gen, genistein; Gly, glycitein; DHD, dihydrodaidzein; DHG, dihydrogenistein; Eq, equol; *O*-dma, *O*-desmethylangolensin (from [94], with the permission from Elsevier Science).

samples were defatted twice with *n*-hexane and hydrolyzed with enzyme or acid. The hydrolyzed samples were extracted with diethyl ether:*n*-hexane (1:1) prior to HPLC separation. The HPLC fractions were then treated with BSTFA or d_9-BSA (silylation) for GC–MS analysis.

A simple GC–MS method for quantitation of isoflavones and lignans in human urine was developed by Grace et al. [107], which requires only a small amount of urine and utilizes only one SPE stage. Triple ^{13}C-labeled internal standards of daidzein, genistein, equol, enterolactone and enterodiol for their corresponding analytes, and anthraflavic acid for glycitein and *O*-desmethylangolensin were used. Urine samples mixed with known amount of internal standards were first hydrolyzed with β-glucuronidase/sulphatase from *H. pomatia*, the hydrolyzate was then extracted via SPE on Strata C18-E cartridges. The resulting extracts were then derivatized with pyridine–BSTFA and TMCS.

Utilizing the isotope deuterated GC–MS method under the selected ion mode (ID-GC–MS–SIM) described previously [92], Boway et al. [102] studied the metabolism of isoflavones and lignans in germ-free rats and in human flora associated rats to search for evidence on role that the gut microflora play in the absorption and metabolism of these phytoestrogens. The conjugates of the phytoestrogen and their metabolites daidzein, genistein, equol, *O*-desmethylangolensin, enterolactone and enterodiol in vitro biological samples were first hydrolyzed and extracted with ethyl ether and further purified by several ion-exchange chromatography prior to silylation for GC–MS analysis.

A sensitive and reliable GC–MS method for determination of bisphenol A and two phytoestrogens daidzein and genistein in infant formula powders were represented by Kuo and Ding [108]. Prior to SPE (C-18), sample solution was ultra-centrifuged, and then silylated by adding the silylating agent BSTFA:TMCS:DTE (1,4-dithioerythritol) (1000:10:2, v/v/w).

Setchell et al. [109] recently investigated the pharmacokinetics of two major soy phytoestrogens daidzein and genistein using 4-^{13}C-labeled tracers in premenopausal women. GC–MS was applied to examine the labeled daidzein and genistein in human plasma using the isoflavone homologue dihydrofavone as the internal standard, while urine samples were measured by LC–MS. Prior to SPE (C-18), blood samples were centrifuged at 3000 × *g* for 10 min at 4 °C and the serum was removed and known amount of internal standard was added. Extracts were then hydrolyzed with *H. pomatia* digestive juice and a mixed β-glucuronidase-sulphatase preparation. After hydrolysis, Sephadex-LH20 chromatography was applied to further purification of the phenolic isoflavones, and the isoflavones were converted to the *tert*-butyldimethylsilyl (*t*BDMS) ether derivatives for GC–MS analysis.

The phytoestrogen precursors in lignan enriched diets derived from rye and wheat, which were used for the

metabolism study with catheterized pig, were analyzed by ID-GC–MS [103]. A GC–MS method, as well as the sample preparation procedures used for analysis of lignan precursors matairesinol, secoisolariciresinol, isolariciresinol, lariciresinol, pinoresinol, and syringaresinol in the pig diets, was modified from the previously published method for analysis of isoflavonoids and lignans in food samples [93].

A comparative study on concentrations of isoflavone phytoestrogens daidzein, genistein and their metabolites equol and *O*-desmethylangolensin in serum from Japanese and British men and women was conducted by ID-GC–MS [106]. Serum samples mixed with known amounts of internal standards ($^2H_6$-matairesinol, $^2H_6$-secoisolariciresinol and $^2H_8$-anhydrosecoisolariciresinol) were first hydrolyzed with β-glucuronidase/aryl sulphatase enzyme, and the hydrolyzate was then chromatographed on Sephdex LH-20. The resulting extracts were derivatized for GC–MS analysis by reaction with BSTFA.

The pharmacokinetic behavior of pure isoflavones in healthy humans has been examined [110]. Plasma concentrations of daidzein, genistein, glycitein, biochanin A, formononetin and equol were quantified by GC–MS using ^{13}C-daidzein, ^{13}C-genistein and 7,4′-dihydroxyflavone as internal standards, which were added to the plasma prior to its extraction and work-up. After extraction (SPE) and enzymatic hydrolysis of the conjugates with a combined sulfatase and glucuronidase enzyme preparation, target compounds silylated with *t*BDMS were then determined.

For GC–MS quantitation, an operation mode of selected ion monitoring has been widely applied to the analysis of phytoestrogens and their metabolites. Under electron ionization (EI) mode, the silylated derivatives generally give intensive peaks of molecular ion or $[M - Me]^+$, which can be used for quantification and identification [4,108]. Applications of GC–MS for the determination of phytoestrogens including subjected sample, analyte, internal standard, GC–MS conditions and validation data in the analysis over recent years are listed in Table 1. We summarized some earlier reports as well [85,92,93], since several latter methods were same as or built upon these initiators.

2.2. High-performance liquid chromatography-based methodology

HPLC separation of phytoestrogen is generally carried out on reversed-phase (RP) column with the mobile phase of methanol (MeOH) or acetonitrile (CH_3CN, ACN) and water containing small amount of acid as modifier. The structure of phytoestrogens and their metabolites mostly contains phenolic hydroxyl groups, which exhibit a weak acidic nature. Thus, applications of acidic modifiers, such as formic acid (HCOOH), acetic acid (AcOH), trifluoroacidic acid (TFA) and phosphoric acid (H_3PO_4) can make the analytes to be easily dissociated in a solvent system, thus enhancing the chromatographic separation, resolution and improvement of peak shape. In addition, while MS detector is applied, the mobile phase modifier of a weak acid, such as HCOOH and AcOH can significantly increase the detected sensitivity by enhancing positive ion formation. Inorganic H_3PO_4 should be avoided as it can lead to the damage of MS ion source components. TFA contains many electrophille fluorine groups, so this additive also serves as a proton receptor and leads to ion suppression. Compared to GC, the primary advantage of HPLC analysis is the ease of utilization and simple sample preparation procedure. The weakness of HPLC separation is in poorer chromatographic resolution. The most commonly used coupling with HPLC has been UV detection. Ultraviolet detection is however non-specific, and cannot achieve a good enough sensitivity. These restricting factors have limited HPLC application for analysis of phytoestrogens in multicomponent plant or biological matrices. Nevertheless, the combination of MS with HPLC (LC–MS) has successfully overcome these disadvantages due to its dramatic potentials of exclusive selectivity and excellent sensitivity. Thus, over the last decade, HPLC coupling with various MS detectors has become the most commonly used technique for determination of phytoestrogens, particularly those in biological fluids. Thus, HPLC separation combined with variety of detections, e.g. UV, laser-induced fluorescence (LIF), ED, MS and NMR have been widely applied to analysis of phytoestrogen precursors in plant or plant derived products and their metabolites in biological matrices.

2.2.1. Ultraviolet detection

Determination of phytoestrogen precursors in plant or plant derived products is often by HPLC with UV or UV-diode array detection (DAD) detectors. As phenolic compounds, the structures of all phytoestrogen and their metabolites have at least one aromatic ring with maximum UV absorption (λ_{max}) ranged from 230 to 280 nm. As such, UV or UV-DAD detection can be very useful.

Chemical investigation on isoflavones in red clover (*T. pratense*) and other three related species was carried out by HPLC/UV/ESI-MS in our group [31]. The separated isoflavones were individually analyzed and identified by their molecular ions and characteristic fragment ion peaks using LC/MSD under MS and MS/MS mode, and in comparison with the standard isoflavones. A total of 31 isoflavones were detected in red clover, including 9 aglycones, 8 glycoside and 14 glycoside malonate derivatives. Several isoflavones were also identified for the first time in related species, *T. repense* L. (white clover), *T. hybridum* L. (alsike clover) and *T. campestre* Schreber (hop trefoil). The quantitative study involved acidic hydrolysis for sample preparation during solvent extraction. Based on reversed-phase HPLC, all 10 isoflavone aglycones, daidzein, formononetin, genistein, pseudobaptigenin, glycitein, calycosin, prunetin, biochanin A, irilone and pratensein in hydrolyzed extracts were successfully separated within 40 min and quantified individually by UV and MS detectors (Fig. 8). Another analytical approach for detection of isoflavones in Edamame and Tofu soybeans has been also achieved by using the slightly modified method

Table 1
Applications of GC–MS for analysis of phytoestrogens

Sample/analyte	Sample preparation	Internal standard	GC condition (column/thermal gradient)	MS conditions	Validation	Reference
Human urine*/HD, HG, 6′-OH-*O*-DMA and *cis*-4-OH-EQ	SPE (C-18), enzymatic hydrolysis, diethyl ether extraction, Sephadex-LH20 chromatography		BP-1 (SGE) capillary column (12.5 m × 0.22 mm i.d., 0.25 μm), initial temperature was 150 °C, held for 1 min, heated 50 °C/min to 220 °C, then raised to 260 °C at a rate of 2 °C/min	EI, full scan		[85]
Human urine/ENL, END, MAT, D, G, EQ and *O*-DMA	SPE (C-18), DEAE-Sephadex (Ac^-) chromatography, enzymatic hydrolysis, SPE (C-18), QAE-Sephadex (Ac^- and $Carb^-$) chromatography	Deuterated standards of corresponding analytes	BP-1 (SGE) vitreous silica capillary column (12.5 m × 0.22 mm i.d., 0.25 μm), initial temperature was 100 °C, held for 1 min, then raised to 280 °C at a rate of 30 °C/min	EI, SIM	Recovery: 96.6–105.5%, CV (within-assay): 0.8–15.2%; (between-assay): 4.1–13.9%	[92]
Plant-derived food/F, C, B-A, D, G, SECO and MAT	Enzymatic hydrolysis, Ether extraction, acidic hydrolysis (water phase), chromatography on DEAE-Sephadex-OH^- and QAE-Sephadex-Ac^-	Deuterated standards of corresponding analytes	GC and MS conditions were the same as described in [92]		Recovery: 95.5–105.5%, CV (within-assay): 3.1–9.6%; (between-assay): 7.0–21.2%	[93]
Human urine*/D, G, GL and metabolites	SPE (C-18), enzymatic hydrolysis, ether extraction, Sephadex-LH20 chromatography	Deuterated standards of corresponding analytes	GC and MS conditions were the same as described in [95]			[94]
Human and rats urine/D, G, EQ, *O*-DMA, ENL and END	Enzymatic hydrolysis, ether extraction, ion-exchange chromatography	Deuterated standards of MAT, SECO and anhydro-SECO	GC and MS conditions were the same as described in [92]			[102]
Plant-derived diets/SECO, MAT, LAR, PINO and SYR	Enzymatic hydrolysis, diethyl ether extraction, acidic hydrolysis (water phase), eby diethyl ether:ethyl acetate		GC/MS analysis was slightly optimized from [92]			[103]
Human serum/D, G, EQ and ENL	Enzymatic hydrolysis, ethyl acetate extraction, Sephadex-LH20 chromatography	Deuterated standards of corresponding analytes	SE54 silica capillary column (12 m × 0.32 mm i.d.), from 190 to 240 °C at a rate of 49.9 °C/min	EI, SIM	LOQ: 0.1 μg/mL	[106]

Table 1 (*Continued*)

Sample/analyte	Sample preparation	Internal standard	GC condition (column/thermal gradient)	MS conditions	Validation	Reference
Flaxseed and pumpkin seeds*/lignans	Defatted by *n*-hexane, enzymatic or acidic hydrolysis, extracted by diethyl ether:*n*-hexane (1:1), HPLC chromatography		MDN-5S fused silica capillary column (28.7 m × 0.25 mm i.d., 0.25 μm), initial temperature was 60 °C, held for 1 min, then raised to 250 °C at a rate of 30 °C/min, held for 10 min, and then raised to 275 °C at a rate of 1 °C/min	EI, full scan (ion trap MSD)		[67]
Human urine/D, G, GL, EQ, *O*-DMA, ENL and END	Enzymatic hydrolysis, SPE (C-18)	^{13}C-labeled D, G, EQ, ENL and END, anthraflavic acid for GL and *O*-DMA	GC-1, 100% dimethyl-polysiloxane column (15 m × 0.25 mm i.d., 0.25 μm), 160–300 °C at a rate of 10 °C/min, held for 1 min	EI, SIM	Recovery: 93.2–104.3% (30 ng/mL spike) and 89.6–99.1% (150 ng/mL spike), CV; (within-assay): 2.0–6.5%, (between-assay): 3.2–26.5%, LOQ: 1.2–5.0 ng/mL	[107]
Infant formula/bisphenol A, D and G	Prior to SPE (C-18), powder milks were ultra-centrifuged	Chrysene-d_{12}	DB-5MS capillary column (12.5 m × 0.25 mm i.d., 0.25 μm), Initial temperature was 100 °C, held for 2 min, raised to 250 °C at a rate of 10 °C/min, raised to 300 °C at a rate of 5 °C/min and then held for 5 min	EI, full scan and SIM	Recovery: 79–83%, R.S.D.: 5% for bisphenol A; 11% for D; 13% for G, LOQ: 1 ng/g for bisphenol A, 10 ng/g for D and G (0.5 g sample)	[108]
Human plasma/[4-^{13}C] D and [4-^{13}C] G	Prior to SPE (C-18), blood samples were centrifuged at 3000 × *g* for 10 min at 4 °C and the serum was removed, enzymatic hydrolysis, Sephadex-LH20 chromatography	Dihydro-flavone	DB-1 fused silica capillary column (12.5 m × 0.25 mm i.d., 0.25 μm), 260–310 °C at a rate of 10 °C/min	EI, SIM	CV (within-assay): 0.5% for D and 1.0% for G, (between-assay): 5.0% for D, 7.0% for G and 10% for EQ (5–7 ng/mL)	[109]
Human plasma/D, G, GL and EQ	SPE (C-18), enzymatic hydrolysis, SPE (C-18), Sephadex-LH20 chromatography	[4-^{13}C] D, [4-^{13}C] G and 7, 4″-dihydroxy-flavone	GC–MS conditions were the same as described in [109]			[110]

Analyte: D, daidzein; G, genistein; GL, glycitein; HD, dihydrodaidzein; HG, dihydrogenistein; F, formononetin; C, coumestrol; B-A, biochanin-A; EQ, equol; *O*-DMA, *O*-desmethylangolensin; ENL, enterolactone; END, elactrodiol; MAT, matairesinol; SECO, secoisolariciresinol, LAR, lariciresinol; PINO, pinoresinol and SYR, syringaresinol.

* Qualitative identification; EI, electron ionization; SIM, selected ion monitoring; SPE, solid-phase extraction; R.S.D., relative standard derivation; CV, coefficient variation; LOQ, limit of quantitation.

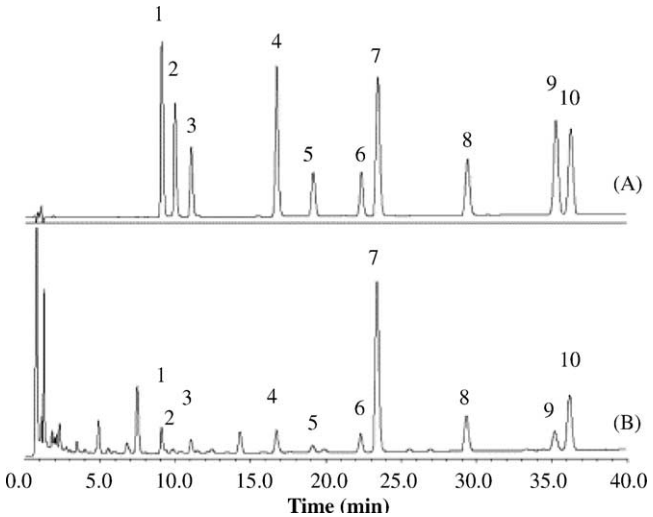


Fig. 8. LC–UV_{254} chromatograms of: (A) a standard mixture and (B) hydrolyzed red clover leaf extract: daidzein (**1**); glycitein (**2**); calycosin (**3**); genistein (**4**); pratensein (**5**); pseudobaptigenin (**6**); formononetin (**7**); irilone (**8**); prunetin (**9**); biochanin A (**10**) (from [31], with the permission from Elsevier Science).

as described previously in our laboratory [31,111]. Positive atmospheric pressure interface (API) MS and MS/MS was used to provide molecular mass information and led to the identification of a total 16 isoflavones, including three aglycones, three glycosides, two glycoside acetates and eight glycoside malonates. The major isoflavones in soybean seeds were daidzein and genistein glycoside and their malonate conjugates. Trace levels of daidzein and genistein acetyl glycosides were found only in the mature dry soybean seeds. To facilitate quantitative analysis, acid hydrolysis during extraction of soy samples was selected to convert the various phytoestrogen conjugates into their respective isoflavone aglycones, allowing accurate quantitation of total phytoestrogens as aglycones. Based on HPLC combined with UV and MS detector, all three soy isoflavone aglycones, daidzein, genistein and glycitein in hydrolyzed extracts were successfully quantified within 25 min with formononetin used as the internal standard.

Maubach et al. [112] developed an HPLC/UV method for the quantitation of daidzein, equol, and genistein in human breast tissue. They conducted a study with two groups of people that ingested either a soy isoflavone preparation or a placebo tablet, and compared with the data collected for urine and serum of the same subjects using standard methods. Biological samples were first hydrolyzed with β-glucuronidase/sulphatase. Prior to SPE (C-18), the breast tissue samples were added a known amount of an internal standard, 4-hydroxybenzophenone (4-HBPH), and were defatted by extraction with *n*-hexane. The blood and urine samples with known amount of internal standard 4-HBPH were directly extracted with ethyl acetate for sample pre-purification. Analytical results revealed that the major daidzein metabolite equol was the predominant phytoestrogen in breast tissue, and the concentrations of phytoestrogens were at least 100-fold higher in urine than in serum and breast tissue.

Duke et al. [113] studied the effect of herbicides on biosynthesis of soy isoflavones. Using HPLC/UV, soybean seeds treated with different herbicides were analyzed for daidzein, genistein, glycitein and their glucosides, as well as herbicide residues. Oven-dried, ground soybeans were directly extracted with 80% MeOH prior to HPLC analysis. No remarkable effects of any treatment were observed on the contents of soy isoflavones. The important of potassium fertilization was also studied on final isoflavone concentrations in soybean [114]. The isoflavone contents of daidzein, genistein and glycitein in different soybean varieties cultivated on soils from low to high exchangeable potassium concentrations were examined by HPLC–UV. Soy samples were hydrolyzed to convert the conjugates to three isoflavone aglycones daidzein, genistein and glycitein, which were assumed to obtained total isoflavone concentrations. Isoflavone concentrations were found to increase in the soybeans produced on low- to medium- potassium soils.

An analytical method for the determination of isoflavone phytoestrogens daidzein, genistein, formononetin and biochanin A in hydrolyzed red clover (*T. pratense*) was developed and validated by using RP-HPLC combined with UV detector [115]. This study involved acidic hydrolysis (TFA) for sample preparation and 6-methoxyflavone was used as the internal standard. Isoflavone stability in soy products were investigated during processing and storage [116]. The loss of isoflavones and the formation of decomposition were detected by HPLC equipped with UV-DAD.

Thomas et al. [117] developed and validated an HPLC–UV method for quantification of the principle soy isoflavones genistein, daidzein and glycitein, and their primary conjugated metabolites in human plasma and urine with 4-HBPH used as the internal standard. For quantitation of free isoflavones in biological fluids, sample preparation simply involved solvent extraction with methyl *tert*-butyl ether. For total isoflavone quantitation in biological fluids, the enzymatic hydrolysis with β-glucuronidase/sulphatase was employed prior to solvent extraction. This analytical approach has been well validated in close agreement with the US Food and Drug Administration's (FDA) guidelines for the validation of methods to be used in support of pharmacokinetic studies.

A simple HPLC/UV method for quantitation of lignans in virgin olive oil was also developed [118]. Samples were treated by using SPE through a diol cartridge, and with both *p*-hydroxyphenylacetic and *o*-coumaric acid used as internal standards. The major lignan precursor, secoisolariciresinol diglucoside was also analyzed in diets containing flaxseed by HPLC coupling with UV-PDA detection [119]. Applications of HPLC combined with UV or DAD including subjected sample, analyte, internal standard, chromatographic conditions, detection and validation data for analysis of phytoestrogens reported in recent years are summarized in Table 2. Several of these studies also used both UV and MS detection [31,111,119,125].

Table 2
Applications of HPLC combined with UV and ED for analysis of phytoestrogens

Sample/analyte/IS	Sample preparation	HPLC and conditions	Detection and conditions	Validation	Reference
Red clover and related species/isoflavones (conjugates and aglycone)	Extracted by aqueous MeOH (80%) (identification), acidc hydrolysis during extraction (quantitation)	Phenomenex prodisyl ODS (**3**) column (150 mm × 3.2 mm i.d., 5 μm), A: 0.1% HCOOH/H_2O; B: 0.1% HCOOH/ACN; gradient: 20–40% B, 0–40 min; flow rate: 1 mL/min and 1/4 was split into MSD	UV-DAD: 254 nm, ion trap MS: ESI in positive ion mode; MS and MS/MS mode for identification, MRM for quantitation	Accuracy: <15%, R.S.D.: <15%, LOD: ∼24 ng/mL (UV); ∼6 ng/mL (MS)	[31]
Flaxseed/lignan	Defatted sample was extracted with aqueous MeOH (80%), acidic hydrolysis	RP-C_{18} column (250 mm × 10 mm i.d., 5 μm), A: 0.2% AcOH/H_2O; B: ACN; gradient: 30–50% B, 0–55 min; 50–30% B, 55–60 min; flow rate: 0.6 mL/min	UV-DAD: 200–400 nm		[96]
In vitro biological sample/ENL, END, MAT, SECO, PINO, SYR, ARC, 7-OH-MAT, ISOL and LAR	Extracted with diethyl ether	Not indicated	ED-CEAD: 575 mV for ENL, END and 7-OH-ENL and 420 mV for other analytes (detection potential)		[66]
Soybean/isoflavones (conjugates and aglycone)/F	Sample preparation was as described in [31]	Same column as [31], A: 0.1% HCOOH/H_2O; B: 0.1% HCOOH/ACN; gradient I: 15–35% B, 0–40 min; flow rate: 0.8 mL/min and 1/4 was split into MSD (identification); gradient II: 20– 35% B, 0–30 min; flow rate: 1 mL/min and 1/4 was split into MSD (quantitation)	UV-DAD: 254 nm, ion trap MS: ESI in positive ion mode; MS and MS/MS mode for identification; MRM for quantitation	R.S.D.: <10% (UV); <15% (MS), LOD: 11.72–15.38 ng/mL (UV); 2.93–3.85 ng/mL (MS)	[111]
Human breast tissue, urine and serum/D, EQ and G/4-HBPH	Enzymatic hydrolysis, defatted by *n*-hexane, SPE (C-18) (breast tissue); extracted by ethyl acetate (fluids)	XTerra^{TM} MS C_{18} column (250 mm × 4.6 mm i.d., 5 μm), isocratic, 40% solvent B (ACN/MeOH, 20:80) in solvent A (0.05% HCOOH/H_2O); flow rate: 1.5 mL/min	UV-PDA: 249 nm for D, 230 nm for G and 261 nm for EQ	Recovery: 70 ± 5.6% (breast tissue); 100 ± 14.1% (fluids), R.S.D.: 0.1–1.4% (intra-assay); 9.3–19.0% (inter-assay), CV: 11.1–16.4%, accuracy: 81.8–86.0%, LOD: 24.7 nmol/l for D; 28.4 nmol/l for G and 148.0 nmol/l for EQ, LOQ: 62.5 nmol/l for D and G, and 125.0 nmol/l for EQ	[112]
Soybean/isoflavones	Extracted with aqueous MeOH (80%)	Zorbax SB-Aq C_{18} reversed-phase column (150 mm × 4.6 mm i.d., 5 μm), A: 0.05% AcOH/H_2O; B: 0.05% AcOH/ACN; gradient: 20% B, 0–2 min; 20–40% B, 2–18 min; 40–100% B, 18–23 min; held 100% B for 3 min; 100–20% B, 26–27 min and then held 20% B for 7 min; flow rate: 0.6 mL/min	UV-PDA: 260 nm		[113]
Soybean/D, G and GL	Acidc hydrolysis during extraction	Nova Pak C_{18} column (150 mm × 3.9 mm i.d., 5 μm), A: 4% AcOH/H_2O; B: MeOH; gradient: 30–35% B, 0–12.5 min; 35–50% B, 12.5–13 min; 50–70% B, 13–15 min; 70–75% B, 15–22.5 min; 75–30%B, 22.5–23 min; flow rate: 1.5 mL/min	UV-PDA: 200–300 nm		[114]

Red clover/D, G, F and B-A/6-methoxy-flavone	Acidc hydrolysis during extraction (TFA)	Hypersil BDS-C_{18} column (250 mm × 4.0 mm i.d., 5 μm), A: sulfuric acid/H_2O (PH 2.7); B: ACN; gradient: 20–37% B, 0–35 min; 37–100% B, 35–45 min; held 100% B for 5 min; 100–20% B, 50–51 min; and then held 20% B for 10 min; flow rate: 1.0 mL/min	UV-PDA: 254 nm	Recovery: 99.2% (B-A); 98.2% (F), R.S.D.: 2.54–5.06%, LOD: 0.6–3.7 ng, LOQ: 2.0–10.0 ng (on column)	[115]
Soy milk/D, G and the glycosides	Mixed with MeOH and centrifuged	SupelcoSil C_{18} reversed-phase column (250 mm × 4.6 mm i.d.), A: 25% MeOH/H_2O; citrate solution (PH 3.5); gradient: 0–50% B, 0–20 min; isocratic eluant to 38 min	UV-DAD: 262 nm		[116]
Human plasma and urine/D, G and GL/4-HBPH	Enzymatic hydrolysis, extracted by methyl *tert*-butyl	A Luna phenyl-hexyl column (150 mm × 4.6 mm i.d., 5 μm)/Zorbax Eclipse XDB-phenyl column (75 mm × 4.6 mm i.d., 3.5 μm) with guard column, A: 0.05 M ammonium format (PH 4.0); B: MeOH/ACN (50:50) or 100% MeOH (isocratic or linear gradient)	UV: 259 nm	Recovery: 92–108% (urine); 37–55% (plasma), R.S.D.: 2.5–8.4%, accuracy: 93–107%, LOQ: 1.76–2.55 ng/mL (plasma); 22.3–22.7 ng/mL (urine)	[117]
Virgin olive oil/PINO and 1-acetoxy-PINO/*p*-OH-PA and *o*-CA	SPE	Lichrospher 100RP-18 column (250 mm × 4.0 mm i.d., 5 μm), A: 3% AcOH/H_2O; B: MeOH/ACN (50:50); gradient: 5–30% B, 0–25 min; 30–35% B, 25–35 min; 35–40% B, 35–40 min; 40–70% B, 40–50 min; 70–100% B, 50–55 min and then held 100% B for 5 min; flow rate: 1.0 mL/min	UV-PDA: 240, 280 and 335 nm	Recovery: >90%, R.S.D.: <6.5%	[118]
Food/SDG	Defatted sample was extracted with aqueous MeOH (70%), base hydrolysis	Symmetry C_{18} column (250 mm × 4.6 mm i.d., 5 μm), A: 1% AcOH/H_2O; B: MeOH; gradient: 5–60% B, 0–44 min; held 60% B for 4 min; 60–5% B, 48–55 min	UV-PDA: 280 nm, MS: ESI in negative ion mode; MS/MS mode for identification	Recovery: 73–75%	[119]
Soybean cultivars/D, G, GL and their glucosides and malonates	Extracted with ACN/HCl, filtrate was dried and redissolved in aqueous MeOH (80%)	YMC AM-303 ODS column (250 mm × 4.6 mm i.d.), A: 0.1% AcOH/H_2O; B: 0.1% AcOH/ACN; gradient: 15–35% B, 0–50 min and held 35% B for 10 min; flow rate: 1 mL/min	UV: 256 nm		[120]
Soy food and soy protein isolate/isoflavones/equilenin	Extracted with aqueous MeOH (80%)	C_{18} reversed-phase column (250 mm × 4.6 mm i.d., 5 μm), A: 90% ACN/H_2O; B: $AcONH_4$/TFA buffer; gradient: 0% B, 0–2 min; 0–50% B, 2–24 min and held 50% B for 5 min; flow rate: 1 mL/min	UV-DAD: 260 nm	R.S.D.: 5.6%	[121]
Pure D and G	Dissolved in MeOH or DMSO	HPLC and conditions were the same as described as [116]	UV-DAD: 262 and 254 nm		[122]
Soy-based food/isoflavone	Prior to SPE (poly-amide), samples were extracted with aqueous MeOH (80%)	Nova Pak C_{18} column (300 mm × 4.6 mm i.d.), mobile phase as described as [124] consisting A: 0.1% AcOH/H_2O and B: 0.1% AcOH/ACN in gradient	UV-DAD	Recovery: 99–103% (D) and 96–103% (G)	[123]

Table 2 (*Continued*)

Sample/analyte/IS	Sample preparation	HPLC and conditions	Detection and conditions	Validation	Reference
Soybean and soybean products/D, G, F, B-A and C	Acidc hydrolysis during extraction	Phenyl Nova Pak column (150 mm × 3.9 mm i.d., 4 μm), mobile phase as described as [126], isocratic system of ACN/H_2O (33:67); flow rate: 1.0 mL/min	UV-PDA: 259.2, 248.6 and 343.1 nm, triple-quadrupole MS: APCI in positive ion mode; tendem MS mode for identification	Recovery: 89–104%, CV: 2.4–6.8%	[125]
Soy based supplement/isoflavone, D, G, GL and their glucosyl, acetyl and malonyl derivatives	Enzymatic and acidic hydrolysis, extracted with diethyl ether (for aglycone quantitation), extracted with 80% aqueous EtOH (for identification)	Inertsil ODS-3 column (150 mm × 3 mm i.d., 3 μm), A: 0.5 mM sodium acetate buffer (pH 5.0):MeOH (80:20); B: 0.5 mM sodium acetate buffer (pH 5.0):MeOH:ACN (40:40:20); gradient: held 30% B for 15 min; 30–50% B, 15–25 min; 50–75% B, 25–35 min; 75–100% B, 35–40 min; held 100% B for 5 min; 100–30% B, 45–47 min; held 30% B for 13 min; flow rate: 0.3 mL/min	ED-CEAD: 8 channels, detection potentials: 510 mV (D and G) and 590 mV (other analytes)	R.S.D.: 1.1–7.6% (intra-assay); 6.3–12.0% (inter-assay), LOD: 38.0–98.3 fmol (on column)	[137]
Human urine/ENL, END, SECO, MAT, LAR, PINO, SYR and ISOL	Enzymatic and acidic hydrolysis, Ether extraction, chromatography on QAE-Sephadex-Ac^-	HPLC column, mobile phase component and gradient elution are same as described in [137] (in gradient); flow rate: 0.3 mL/min	ED-CEAD: 8 channels, detection potentials: 640 mV (ENL and END), 300 mV (SECO and SYR) and 420 mV (LAR, PINO, MAT and ISOL)	Recovery: 81.8–102%, CV: 2.1–8.72%, LOD: 0.61–1.41 ng/mL, LOQ: 3.05–7.05 ng/mL	[138]
Flaxseed/MAT/bisphnol A	Defatted with *n*-hexane, acidic hydrolysis during extraction	Lichrospher 60 RP-Select B column (250 mm × 4 mm i.d., 5 μm), A: sodium acetate buffer (PH 3.0) B:ACN (3:1); flow rate: 0.8 mL/min	ED-CEAD: 8 channels, detection potentials: 250 mV (MAT) and 600 mV (bisphenol A)	Recovery: 93.1 ± 2.8% (MAT) and 96.8 ± 1.3% (bisphenol A), CV: 2.2% (MAT), LOQ: 5 μg/g	[139]
Human blood/D, G and EQ	Enzymatic hydrolysis, extracted with MeOH/ACOH (100:5)	TSK-gel ODS-80TS QA column (150 mm × 4.6 mm i.d., 5 μm), mobile phase: water/MeOH/AcOH (57:41:2) containing 50 mmol lithium acetate; flow rate: 0.9 mL/min	ED: 950 mV (detection potential)	LOD: 0.01 μg/mL	[140]

The ratio of mobile phase components is expressed as v/v. IS, internal standard; ESI, electrospray ionization; SIM, selected ion monitoring; MRM, multiple reaction monitoring; DAD, diode array detection; PDA, photodiode array detector; CEAD, coulometric electrode array detector; SPE, solid-phase extraction; R.S.D., relative standard derivation; CV, coefficient variation; LOQ, limit of quantitation; LOD, limit of detection; TFA, trifluoroacidic acid. *Analyte*: D, daidzein; G, genistein; GL, glycitein; F, formononetin; C, coumestrol; B-A, biochanin-A; EQ, equol; *O*-DMA, *O*-desmethylangolensin; ENL, enterolactone; END, elactrodiol; MAT, matairesinol; SECO, secoisolariciresinol; SDG, secoisolariciresinol diglucoside; LAR, lariciresinol; ISOL, isolariciresinol; PINO, pinoresinol, SYR, syringaresinol; PA, phenylacetic and CA, coumaric acids; ARC, arctigenin; 4-HBPH, 4-hydroxybenzophenone.

2.2.2. Electrochemical detection

Phenolic compounds, including the phytoestrogen isoflavones, lignans and their metabolites are easily oxidized and thus, generally electroactive. Electrochemical detection is particularly useful therefore in the detection of phytoestrogens, and the sensitivity is generally better than that of UV or DAD for HPLC applications [127–131]. A multichannel electrochemical detector, so called coulometric electrode array detector (CEAD) has been widely used for determination of phytoestrogens as it can provide good selectivity and permit resolution of co-eluting analytes based on differences in their oxidation or reduction behavior [132–136]. This detector is designed based on the use of multiple electrode pairs, which are maintained at different potentials. Phenolic phytoestrogens are oxidized and the oxidation potential ranges from 0 to +1000 mV. In general, maximum potentials are kept close to or under + 700 mV [137]. Due to the properties of sensitivity, selectivity and resolution, this technique expands the analytical capabilities for multiple component analysis and can minimize sample pretreatment especially for complex biological fluids.

Nurmi et al. [137] reported an HPLC method for quantitation of total isoflavones as aglycones in soy-based products after hydrolysis, and identification of the different forms of the isoflavone conjugates with CEAD. Samples were first hydrolyzed with β-glucuronidase and then the hydrolyzate was extracted with diethyl ether. The sample in the water phase was further hydrolyzed with 2 M HCl and extracted again with ether. A multichennel electrochemical detector was applied to determination of isoflavone aglycones and identification of the conjugated forms at detection potentials + 510 and + 590 mV. A slightly modified HPLC–CEAD method was also developed to measure both plant and mammalian lignans in human urine [136–138]. In this study, sample pretreatment first involved repeated enzymatic and acidic hydrolysis, and the hydrolyzate was then extracted with diethyl ether. The resulting extracts were treated with QAE-Ac^- ion exchange chromatography prior to HPLC separation. This method was validated and the results indicated that this technique could be applied to normal urine containing low amounts of plant lignans and moderate amounts of mammalian lignans. The HPLC–CEAD chromatograms of a standard mixture and urine sample under different detection potentials modified from original report are presented in Fig. 9.

Kraushofer and Sontag [139] described an HPLC method coupled with CEAD for the detection of phytoestrogen lignan precursor matairesinol in flaxseed using bisphenol A as the internal standard. The defatted samples were hydrolyzed with concentrated HCl during solvent extraction. Optimal potentials were set on +250 mV for matairesinol and +600 mV for bisphenol A and validation of this method was also present. Zubik and Meydani [140] investigated the bioavailability of the soy isoflavones daidzein and genistein in American women after ingestion of the aglycone or glucoside form of isoflavones. In this study, HPLC–ED was used to monitor the availability of daidzein, genistein and

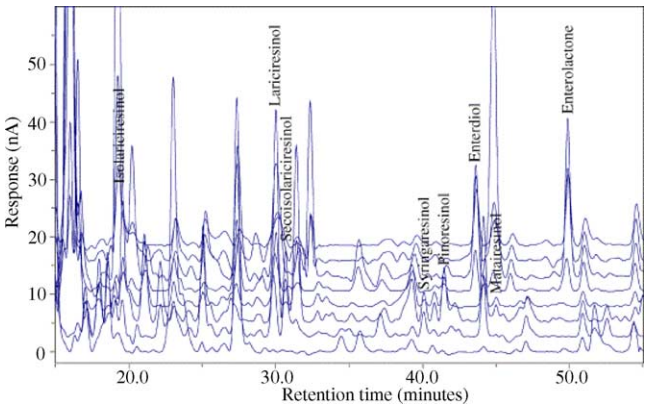


Fig. 9. HPLC–CEAD (eight channels) chromatogram of a human urine sample illustrating six lignan precursors and two mammalian lignans (from [138], with the permission from Elsevier Science).

equol in the subjected blood sample. Plasma was first hydrolyzed with β-glucuronidase/sulfatase preparation to release the free forms of isoflavones from the glucuronide and sulfate conjugates. Genistein and daidzein were then extracted with methanol:acetic acid (100:5, v/v), mixed and sonicated, then centrifuged for 5 min. Detection potentials were set on +950 mV for both analytes.

Investigation on the in vitro metabolism of plant lignans was examined by Heinonen et al. [66]. In their study, the quantitative analyses of lignan precursors and the mammalian lignans enterolactone and enterodiol were analyzed using HPLC combined with coulometric electrode array detector (CEAD). Prior to HPLC separation, the sample was directly extracted with diethyl ether. Detection potentials were set on +420 and 575 mV for different analytes. Applications of HPLC combined with ED or CEAD for analysis of phytoestrogens including subjected sample, analyte, internal standard, chromatographic conditions, detection and validation data reported in recent years are also summarized in Table 2.

2.2.3. Fluorescence detection

Fluorescence detection is generally more sensitive and selective than UV absorption. Many classes of analytes are natively fluorescent, but often only upon absorption of short-wavelength UV light. This has restricted its applicability. However chemical derivatization with a suitable fluorescent label such as Al^{3+} can match the excitation wavelength and this approach has broadened the application [141,142]. Richelle et al. [143] more recently investigated the difference of human bioavailability for original and enzymatic hydrolyzed isoflavone enriched soy preparations. This study involved the utilization of HPLC with fluorescence detection to examine the presence of major soy isoflavones daidzein, genistein, glycitein and their metabolites in human plasma and urine. Plasma and urine samples were first hydrolyzed with a combined sulfatase and glucuronidase enzyme preparation. The hydrolyzates were then chromatographed through a Baker SPE column prior to HPLC separation. HPLC was performed on Nucleosil 120-3 C_{18} column using the mobile

phase consisting solvent A (2 g phosphoric acid in 999.5 g water) and B (100% ACN) in gradient at flow rate of 0.8 mL/min. Analytes were simultaneously detected by UV for daidzein, glycitein, genistein, dihydrogenistein and dihydrodaidzein, and a fluorescence detection at 280 nm excitation and 310 nm emission for equol.

2.2.4. Mass spectrometry

Combined liquid chromatographic and mass spectrometric techniques (LC–MS) have been contributing in a decisive way to the progress of phytoestrogen analysis, particularly of that in complex biological matrices. Investigation upon the coupling of LC and MS can date back to the early 1970s. In the first two decades, the focus had been towards solving the interface problems and associated new technology [144]. In 1980s and early 1990s, thermospray (TSP) interface was primarily used in many LC–MS analyses. However, because of practical limitations of instrumental robustness and ion source stability, this interface has now been replaced by the new atmospheric pressure ionization (API) methods, which predominantly comprise electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) [145–149].

Electrospray ionization is a "soft" ionization technique and produces little fragmentation of analyte. This interface generally forms protonated $[M + H]^+$ or de-protonated $[M - H]^-$ ions for positive or negative mode MS, respectively. Occasionally simple adduct ions $[M + Na]^+$ or $[M + NH_4]^+$ and $[M + H - H_2O]^-$ could also be occurred. These ion forms or their further reduced daughter ions under MS/MS mode are very useful for analyte identification and quantitation. Under ESI mode, high molecular molecules such as proteins and peptides may be multiply charged. However, for analytes with small molecular weights such as under about 1000 Da, like phytoestrogens and their metabolites, usually single charged ions are predominantly produced. All phytoestrogens and their biological metabolites contain phenolic hydroxyl group with high polarity. With the development of ESI with LC–MS, this technique has been proved to be highly sensitive and greater ionization stability, especially for polar compounds, and very compatible with solvent system components, e.g. MeOH and/or ACN and water with a small amount of acid used for RP-HPLC. As such, ESI technique has been presently dominating the field of MS detection for the determination of phytoestrogens. APCI, on the other hand, is mainly used for relative less polar compounds than ESI, and can generally produce singly charged ions up to about 1500 Da. This technique has a wider dynamic concentration range, but is not suitable for thermally unstable analytes. Additionally unlike ESI, APCI can produce unexpected fragmentation.

Due to the relatively low cost, physically small size, ideal mass range and reasonable scanning speed, quadrupole or triple quadrupole and ion trap MS analyzers equipped with ESI and/or APCI interfaces have dominated the methodology in recent years for phytoestrogen analysis, particularly in complex biological fluids. Compared to magnetic sector instruments, the quadrupole mass filter functions in an entirely different way and offers a variety of advantages. Four precisely parallel rods or poles are arranged in a square, among which each pair of opposite is electrically connected. Ions are propelled from the ion source to the quadrupole analyzer by a small accelerating voltage (about 5 V, while several thousand volts for magnetic sector instrument). Mass separation is achieved depending on ion transmission through the electric fields produced by the electrically connected parallel rods or poles. While field strengths are systematically changing (scan), at any point in the scan, only ions of one mass can pass through the quadrupole system and reach the detector. Ions that are not focused collide with the rods and are destroyed. Quadrupole MS filter has a variety of advantages over magnetic sector instrument. It is more robust, less expensive, physically smaller and more ready to interface with different inlet systems. These have device easy to be constructed as a bench-top instrument. The main disadvantage of this technique is that the mass resolution of single quadruple is not sufficiently high and that useful ion fragmentation cannot be achieved.

The power of the MS analyzer can be increased by coupling the same or different types of mass filters together, so called multiple MS analyzers. Triple quadrupole detector currently is the most commonly used multiple MS instrument, among which the first MS filter is applied to selecting interested ions with specific mass-to-charge value (m/z). Each selected ion is allowed, or induced to fragment in the second quadrupole, and then the resulting fragment ions are analyzed in the third analyzer. This system allows to producing MS/MS spectrum, and provides more structural information and highly selective detection for phytoestrogen identification and quantitation, respectively. Additionally, the development of selected ion monitoring or multiple reaction monitoring (MRM) achieves a high sensitivity for phytoestrogen quantification.

While ion trap instruments do not look like quadrupole-based systems, they are actually an extension of the same technology. The pair of end caps and ring electrodes can be considered corresponding to the four electric rods of quadrupole filter. Due to the capability of performing multiple stage MS (MS^n), ion trap mass spectrometer (IT-MS) has been widely applied, especially in structure determination. Using a coupling of IT-MS with HPLC, phytoestrogenic isoflavones in red clover and related species were recently examined [31]. The isoflavone structures were individually elucidated according to their molecular ions and simply reduced fragments, e.g. de-malonyl and/or de-glycosyl ions under MS mode, and specific RDA fragment ions under MS/MS mode for aglycone confirmation (Fig. 10). A slightly modified method using IT-MS was also applied for analysis of isoflavones in immature fresh and mature soyseed [111].

Single quadrupole, triple quadrupole or ion trap MS analyzers cannot yield accurate mass. Over the last few years, applications of time-of-flight MS (TOF-MS) have been rapidly increasing, due to its high resolution improved by the use of

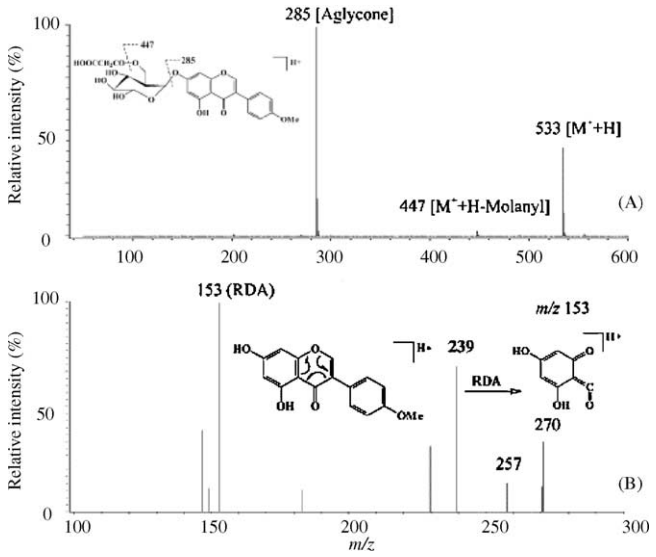


Fig. 10. MS spectrum of biochanin A-7-*O*-β-D-glucoside-6″-*O*-malonate from red clover (A, inset is the structure and its MS fragment pathway), and MS/MS spectrum of biochanin A with precursor ion of *m/z* 285 (B, inset is the structure and its MS/MS fragment pathway. Ion peak at *m/z* 153 is RDA fragment ion, *m/z* 270 is $[M + H - Me]^+$, *m/z* 257 is $[M + H - CO]^+$ and *m/z* 239 may be $[257 - H_2O]^+$) (modified from [31], with the permission from Elsevier Science).

an ion refection device. This resulting increased resolution allows to yielding high mass accuracy for small or medium size molecules and elucidate the empirical formula. TOF analyzer separates ions by making their different velocities after acceleration through a potential, and the time taken for a specific ion to reach the detector is proportional to the square root of its *m/z* value. Tandam mass (MS/MS) spectrometer of TOF can be achieved by coupling with another mass analyzer, which is called hybrid instrument. The most widely used hybrid is presently the coupling of quadrupole with time-of-flight MS (Q-TOF-MS), which can also provide accurate mass data for fragment ions under MS/MS mode. This could be greatly useful for understanding MS/MS fragmentation. However, this instrument cannot be applied under the operation mode of selected ion monitoring.

The coupling of matrix-assisted laser desorption ionization is another commonly used time-of-flight based system (MALDI-TOF). Except for the achieved high mass resolution, this technique is particularly suitable for analysis of huge molecular molecules such as proteins and peptides due to the soft desorption ionization. More recently, the application of MALDI-TOF-MS has gradually replaced the use of fast atom bombardment mass spectrometry (FAB-MS), which was originally employed for study of high molecular mass biological material [145,150,151]. However, MALDI-TOF at present is not a routinely compatible connection to liquid chromatographic system, and at this time it is not a quantitative approach, rather it is an excellent qualitative tool. Whereas, the quantitative measurements using this approach were recently attempted by Sporns and colleagues [152–155]. Wang et al. reviewed the application of phytoestrogen analysis by MALDI-TOF-MS [8].

Fourier-transform ion-cyclotron resonance mass spectrometry (FT-ICR-MS) is another technique of very high resolution, but the instrumentation is large, complex and expensive. Recently, several reviews focusing on various areas of technical aspects and applications of the coupling of LC and MS were present [144,145,149,156–158]. In this section, only LC–MS applications for phytoestrogen analysis over recent years are discussed.

Lagana et al. [159] reported a method for assessing the occurrence of trace level estrogenic compounds in sewage and surface waters using liquid chromatography coupled with tandem mass spectrometry (LC–MS–MS). In their study, 12 representative substances including three phytoestrogens daidzein, genistein and biochanin A were selected for monitoring, as well as endogenous estrogens, alkylphenols, and synthetic hormone mycoestrogen and their metabolites using zearalanone and flavone as internal standards. Solid-phase extraction was performed with OASIS cartridges for sample preparation, and different MS interfaces (ESI and APCI) and ion sources (positive and negative) were utilized for analysis of appropriate analytes. Quantitative analysis was operated under multiple reaction monitoring with protonated or deprotonated ions for individual analytes. Validation of this approach achieved good recovery of above 80% and precision of below 8% for all analytes, and limit of detection (LOD) ranged from 0.1 to 182.0 ng/L in various effluents.

An investigation into the pharmacokinetics of two major soy phytoestrogens daidzein and genistein using 4-^{13}C-labeled tracers in premenopausal women was conducted by Setchell et al. [109]. LC–MS with ESI interface was applied to determine the labeled daidzein and genistein in human urine using dihydrofavone as internal standard under positive ion mode, while plasma samples were measured by GC–MS. For sample preparation, urine samples were hydrolyzed with *H. pomatia* digestive juice and a mixed β-glucuronidase-sulphatase preparation after the addition of internal standard prior to SPE (C-18). Validation of this method resulted in the within-day instrument reproducibility of 3% for daidzein and 4% for genistein and between-batch reproducibility of 8% for daidzein and 10% for genistein ($n = 12$, over 6 months period) between 200 and 300 ng/mL. A slightly modified LC–MS method was also developed for the pharmacokinetic study of soy isoflavones daidzein, genistein and daidzein metabolite equol in human urinary excretions with the stable isotopically labeled internal standards by this laboratory [160].

A sensitive and high throughput LC–ESI-MS and LC–ESI-MS/MS method for quantifying isoflavones daidzein, genistein and metabolite equol in blood from experimental animal and human studies was developed by Twaddle et al. [161]. In this study, sample preparation involved SPE after enzymatic deconjugation, and deuterated standards of each corresponding analytes were added as internal standard. Resulting samples were injected onto the LC–ESI-MS or MS/MS system (under positive ion mode)

for detection using SIM or MRM operation, respectively. Validation of this method was assayed by repetitive analysis of spiked blank serum. The intra- and inter-day accuracy (88–99%) and precision (R.S.D. from 3 to 13%) of measurement were obtained. The lower limit of quantification (LOQ) for all analytes was approximately 0.005 μ*M* using MS/MS detection, and 0.03 μM using MS for genistein and daidzein.

Franke et al. [162] recently reported a new liquid chromatography coupling with a photodiode array detector and mass spectrometer (LC-PDA–MS) assay for examining a variety of phytoestrogens including isoflavones and metabolites, flavones and mammalian lignans in human plasma and urine. Biological fluids were first hydrolyzed with enzymatic preparation and then the hydrolyzates were extracted three times with ethyl ether after addition of known amount of internal standards of taxifolin for quercetin and formononetin for other analytes. The unconjugated analytes were analyzed without enzymatic hydrolysis and directly extracted with diether ether or ethyl acetate. Data was acquired using ESI-MS detector in negative ion mode under selected reaction monitoring (SRM). Validation of this method was assessed by determining precision, spiking recovery and limit of quantitation (LOQ) using plasma and urine specimen.

Clarke et al. [163] developed a method for analysis of the free phytoestrogens and their conjugates in human urine using liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS). Various natural phytoestrogen isoflavones and their major metabolites, mammalian lignans and coumestrol were included in this assay and stable isotopically labeled [$^{13}C_3$] daidzein and [$^{13}C_3$] genistein were used as internal standards for isotope dilution mass spectrometry (ID-MS). Free aglycones and conjugates of isoflavones and lignans were observed in naturally incurred urine samples. For determining free phytoestrogens, urine was injected directly onto the analytical column, whereas enzymatic hydrolysis was applied for analysis of hydrolyzed analytes. Validation assay revealed that the limits of detection

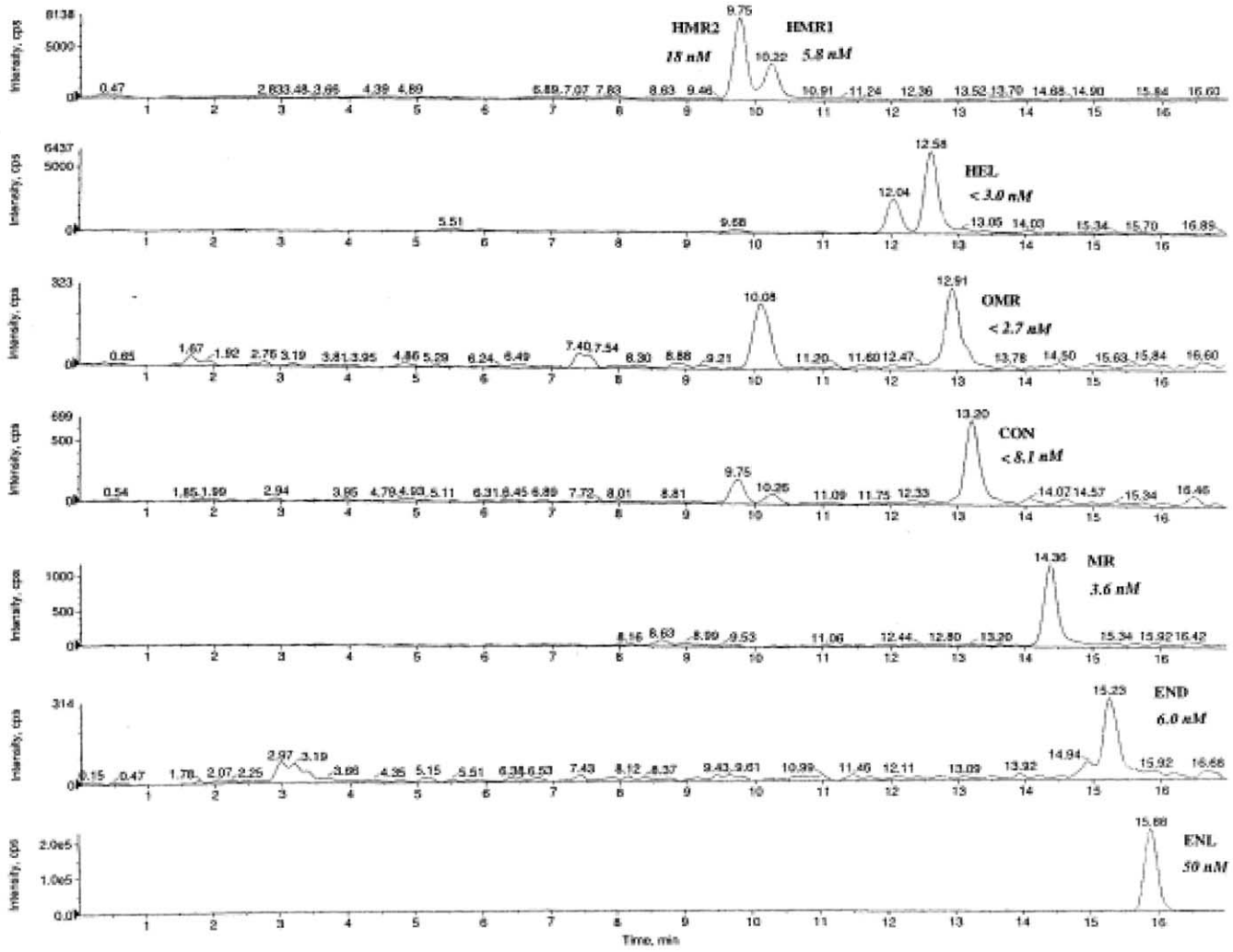


Fig. 11. Extracted ion MRM chromatograms of the lignans in one of the individual hydrolyzed plasma samples: HRM, 7-hydroxymatairesnol; HEL, 7-hydroxyenterolactone; ENL, enterolactone; END, enterodiol; MR, matairesinol; OMR, 7-oxomatairesinol; CON, α-conidendrin (from [165], with the permission from Elsevier Science).

were generally <50 ng/ml, precision was generally <10% CV for conjugates and accuracy of total hydrolyzed daidzein and genistein were within 12%.

The metabolism of lignan precursors secoisolariciresinol diglycoside, secoisolariciresinol, matairesinol and 7-hydroxymatairesinol was examined in vivo by Saarinen et al. [164]. High-performance liquid chromatography combined with electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS) was applied for measuring the lignan metabolites in animal urine under negative mode and flavone was used as internal standard. Urine samples were first enzymatically hydrolyzed and then treated through SPE prior to HPLC injection. Multiple reaction monitoring was optimized as operation mode for quantitation with the molecular ion as precursor ion. An improved LC–ESI-MS/MS method on determination of human plasma lignans was also reported by this laboratory [165]. In that study, plant lignan 7-hydroxymatairesinol and its potential metabolites matairesinol, oxomatairesinol, α-conidendrin, 7-hydroxyenterolactone, enterodiol and enterolactone in human plasma were examined using d_6-enterolactone (for mammalian lignans enterodiol and enterolactone) and d_6-matairesinol (for other analytes) as internal standards (Fig. 11). This method showed good chromatographic linearity, analysis repeatability and SPE recovery.

Grace et al. [166] described an isotope dilution liquid chromatography/tandem mass spectrometric method (ID-LC–MS/MS) for quantification of phytoestrogenic isoflavones and lignans in human serum. Using this established method, three isoflavones daidzein, genistein, and glycitein, two daidzein metabolites *O*-desmethylangolensin and equol, and two mammalian lignans enterodiol and enterolactone in human serum were examined using $^{13}C_3$-labeled internal standards. MS data was acquired under negative ion mode with selected reaction monitoring. A simple, highly automated sample preparation procedure requires small amount of biological fluids and utilizes only one SPE stage, in addition to enzymatic hydrolysis. Validation of this method achieved excellent recoveries varied from 92.5 to 100.7% for the 1 ng/mL spike and from 92.3 to 97.6% for the 50 ng/mL spike, and reproducibility of intra-assay CV 2.8–5.6% and inter-assay CV 3.0–4.4% for all seven analytes. Limits of detection are in the region of 10 pg/mL for all analytes except equol, which had a LOD of approximately 100 pg/mL. Applications of HPLC combined with various MS detectors including subjected sample, analyte, internal standard, chromatographic conditions, MS and MS conditions, and validation data for analysis of phytoestrogens reported in recent years are summarized in Table 3.

2.2.5. Nuclear magnetic resonance spectroscopy

The coupling of high-performance liquid chromatography with nuclear magnetic resonance spectroscopy (LC–NMR) is a further powerful hyphenated technique for the separation and structural elucidation of unknown components in complex matrices. MS detection might achieve molecular weight and fragmentation, and even molecular formula by using high resolution mass spectrometry (HRMS), yet by itself is insufficient to unambiguously assign the molecular structures of an unknown compound, especially those indistinguishable isobars and isomers. NMR spectroscopy is well known as the most powerful spectroscopic technique for obtaining the detailed structural information of molecules, and therefore, of use to discern the structural differences of compounds of the same molecular mass (isobars), or the same molecular formula (isomers), including constitutional, geometrical, and optical isomers.

Direct on-line coupling of NMR with HPLC was reported 20 years ago [182–184]. The application of this technique had been seriously hampered at first due to the lack of sensitivity. However, in the recent decade, the employment of high field magnets (500 MHz and greater up to 800 MHz), the improvement of new solvent suppression techniques (traditional presaturation and WET techniques) [185] and development of probe technology (flow probe) have dramatically enhanced the sensitivity of NMR detection, and made LC–NMR more practical and popular today. Using this modern technique, typical reversed phase HPLC generally involves using the mobile phase of non-deuterated organic solvents, e.g. MeOH and ACN, and relatively inexpensive deuterated water (D_2O). Several reviews regarding various technical aspects and applications of LC–NMR for natural product analysis and drug discovery have been published [186–190].

Fritsche et al. [191] described the separation and characterization of phytoestrogenic lignan precursors secoisolariciresinol diglucoside isomers in flaxseed by on-line LC–NMR–MS. In this study, HPLC separation was carried out on LiChrospher C-18 reversed phase column (250 mm × 4.6 mm i.d., 5 μm) with mobile phase consisting CH_3CN and D_2O in gradient at a flow rate of 1.0 mL/min. UV chromatogram was recorded at 280 nm, and ESI-MS was applied to achieve the molecular mass information including the molecular weight of target analytes under both positive and negative ion mode (Fig. 12). The on-line ^1H NMR experiment was conducted using 600 MHz NMR spectrometer equipped with an LC inverse gradient probe with detection volume of 120 μL, and solvent suppression was performed using the WET sequence. Fig. 13 gives the on-line ^1H NMR spectra of the two diastereoisomers of secoisolariciresinol diglucoside corresponding to peaks 1 and 2 in Fig. 12. According to the acquired chemical shifts and comparing with the previously reported NMR spectral data, the structures of two isomeric forms of secoisolariciresinol diglucoside were successfully elucidated using this approach. The other successful applications of on-line NMR on identification of lignans and isoflavones from plant origins have been demonstrated [192–194] and summarized by others [189,190].

2.3. Capillary electrophoresis-based methodology

Because of the inherent advantages of high separation resolution, low sample quantity requirement, and short assay

Table 3
Applications of HPLC combined with MS detection for analysis of phytoestrogens

Sample/analyte	Internal standard	Sample preparation	HPLC and conditions	MS and conditions	Validation	Reference
Sewage and surface water/phytoestrogen (D, G, B-A, BPA and NP), natural and synthetic hormones	Zearalanone and flavone	Extracted with aqueous MeOH, SPE (OASIS HLB cartridge)	LC-18 packing Alltima column (250 mm × 4.6 mm i.d., 5 μm) and supelguard pre-column (20 mm × 4.6 mm i.d.), A: H_2O or H_2O/10 mM TFA/B: ACN or ACN/10 mM TFA, different gradient programs applied; flow rate: 1 mL/min and 0.2 ml effluent introduced into MSD	PE-Sciex API365 TQ-MS, APCI, NI (synthetic estrogens), ESI, PI (D, G and B-A), ESI, NI (estrogens, BPA and NP), MRM	Recovery: >80%, R.S.D.: <8%, LOD: 0.1–182.0 ng/L	[159]
Human urine/[4-^{13}C] D and [4-^{13}C] G	Dihydro-flavone	Enzymatic hydrolysis, SPE (C-18)	RP-C_{18} column (250 mm × 4.6 mm i.d.), A: 10 mmol $AcONH_4$/1 L H_2O (0.1% TFA); B: ACN; gradient: 0% B for 2 min; 0–50% B, 2–24 min; held 50% B for 5 min; flow rate: 1.0 mL/min	Micromass Quattro LC/MS, ESI, PI, SIM	R.S.D.: 3% for D and 4% for G (intra-assay); 8% for D and 10% for G (inter-assay)	[109]
Human urine/D, G and EQ	^{13}C-labeled D, G and EQ	SPE (C-18), enzymatic hydrolysis	HPLC and conditions were same as described in [109]	MS and conditions were same as described in [109]		[160]
Human and animal blood/D, G and EQ	Deuterated standards of corresponding analytes	Enzymatic hydrolysis, SPE	Ultracarb ODS column (150 mm × 2.0 mm i.d., 3 μm), A: 0.1% AcOH/H_2O; B: ACN; isocratic elution of 35% B; flow rate: 0.2 mL/min	Micromass Quattro Ultima TQ-MS, ESI, PI, SIM (MS) MRM (MS–MS)	R.S.D.: 3–13%, accuracy: 88–99%, LOQ: ∼0.005 μM (MS–MS), 0.03 μM (MS)	[161]
Human urine and blood/isoflavones, flavones and mammalian lignans	F and toxifolin	Enzymatic hydrolysis (omitted for free analytes), extraction with ethyl ether	HydroBond PS C-18 RP-column (100 mm × 3 mm i.d., 5 μm) and HydroBond PS C-18 pre-column (25 mm × 3.2 mm i.d., 5 μm), MeOH–CAN–H_2O (containing 0.5% AcOH or not); different gradient programs applied; flow rate: 0.25 mL/min	Thermo Finnigan LCQ Classic IT-MS, ESI, NI, SRM, UV-PDA: 280 nm	Recovery (different spike level): 60–120% (urine); 77–117% (plasma) CV: very variant, LOQ: 1–201 pg (urine); 1–148 pg (plasma)	[162]
Human urine/isoflavones, coumestrol and mammalian lignans	[$^{13}C_3$] D and G	With or without selective enzymatic hydrolysis	YMC-ODS AM column (250 mm × 3.2 mm i.d., 5 μm), A: 0.1% AcOH/H_2O; B: 0.1% AcOH/ACN; gradient: initial B was 10%, held for 5 min, 10–22% B, 5–20 min; 22–30% B, 20–35 min; 30–46% B, 35–36 min; 46–80% B, 36–56 min; 80–85% B, 56–60 min; 85–10% B, 60–65 min; held 10% B for 10 min; flow rate: 0.5 mL/min	PE Sciex API 2000LC–MS/MS, ESI, NI, MRM, UV: 262 nm	CV: <10%, accuracy: <12%, LOD: <50 ng/mL	[163]

Animal urine/ENL, END, SECO and 7-OH-MAT	Flavone	Enzymatic hydrolysis, SPE (C-18)	Waters symmetry C_{18} column (100 mm × 2.1 mm i.d., 3.5 μm) equipped with Waters Sentry guard column, A: MeOH/0.1% AcOH/isopropanol (99:9.9:0.1); B: 0.1% AcOH/isopropanol (99:1); gradient: 78–27% B, 0–16 min; 27–5% B, 16–17 min; held 5% B for 2 min; 5–78% B, 19–20 min; held 78% B for 5 min; flow rate: 0.2 mL/min	PE Sciex API3000 TQ-MS, ESI, NI, MRM		[164]
Human plasma/ENL, END and lignan precursors	D_6-ENL and d_6-MAT	Enzymatic hydrolysis, SPE (C-18)	HPLC and conditions were slightly modified from [164]	MS and conditions were same as described in [164]	Recovery: 65–101%, R.S.D.: <20%, LOD: 0.13–267 ng/mL	[165]
Human/D, G, GL, *O*-DMA, EQ, ENL and END	[$^{13}C_3$] D, G, *O*-DMA, EQ, ENL and END	Enzymatic hydrolysis, SPE	Xterra MS C_{18} column (150 mm × 2.1 mm i.d., 3.5 μm) equipped with guard column (10 mm × 2.1 mm i.d., 3.5 μm), A: 40% MeOH/0.1% $AcONH_4$; B: MeOH; gradient: 0–25% B, 0–1 min; 25–65% B, 1–7 min; flow rate: 0.2–0.25 mL/min	Micromass Quattro Ultima TQ-MS, ESI, NI, SRM	Recovery: 92.5–100.7% (1 ng/mL spike); 92.3–97.6% (50 ng/mL spike), CV: 2.8–5.6% (intra-assay); 3.0–4.4% (inter-assay)	[166]
Pomegranate fruit*/luteolin, quercetin and kaempferol		Extraction with water prior to ethyl acetate fractionation, or extraction with aqueous MeOH, acidic hydrolysis, SPE (C-18)	Prodigy ODS (3) column (100 mm × 3.2 mm i.d., 5 μm), mobile phase and gradient: 20–35% ACN/2 mM ammonium acetate in 8 min and followed by a 7 min post gradient	Thermo-Finnigan DECA tendem MS, APCI, NI		[167]
*Soybean/daidzin, genistin, glycitin and malonyl genistin	2,5-di-OH-benzal-dehyde	Extraction with EtOH, MeOH or ACN with several water percentage	RP-18 column (Lichrospher 100, 5 μm), A: 0.1% AcOH/H_2O; B: 0.1% AcOH/ACN; gradient: 15–30% B, 0–10 min; held 30% B for 15 min; 30–100% B, 25–35 min; flow rate: 0.3 mL/min	Finnigan LCQ MS, ESI, PI (for identification), UV-PDA: 254 nm (for quantitation)		[168]

Table 3 (*Continued*)

Sample/analyte	Internal standard	Sample preparation	HPLC and conditions	MS and conditions	Validation	Reference
Soy cell cultures*/D, G and glycoside derivatives		Extracted with aqueous MeOH (80%)	Nucleosil 100-C_{18} column (250 mm × 2.1 mm i.d., 5 μm), A: 0.5% AcOH/H_2O; B: 0.5% AcOH/ACN; gradient: 10% B for 5 min; 10–90% B, 5–30 min; held 90% B for 5 min; flow rate: 0.3 mL/min	Micromass Quattro-LC/MS, ESI, PI		[169]
In vitro biological sample*/D, dihydro-D, EQ and *O*-DMA		Extracted with ethyl acetate	Prodigy ODS (3) column (250 mm × 2.0 mm i.d., 5 μm), A: H_2O; B: ACN; gradient: 20–80% B, 0–40 min; flow rate: 0.2 mL/min	ESI, NI		[170]
In vitro biological sample/[^{3}H] and [^{13}C] genistin		Extracted with ethyl acetate or SPE (C-18)	Hypersil Elite C_{18} column (150 mm × 2.1 mm i.d., 5 μm), A: 0.1% AcOH or 15 mM $AcONH_4$/H_2O; B: ACN; gradient: 15–45% B, 0–20 min; flow rate: 0.3 mL/min and 50% effluent introduced into MSD	PE-Sciex API365 TQ-MS, ESI, PI (hydrolyzed sample), Finnigan LCQ IT-MS, APCI, NI (metabolism sample)		[171]
Soy-based food/D, G, Gl and glycoside derivatives	[$^2H_4$] D	Extracted with aqueous EeOH (80%)	YMC ODSAM 250 AS C-18 column (250 mm × 3.2 mm i.d., 5 μm) fitted with a 30 mm × 3.2 mm i.d. guard column, A: 0.5% AcOH/H_2O; B: 0.5% AcOH/ACN; gradient: 15–22% B, 0–30 min; 22–35% B, 30–50 min; 35–45% B, 50–52 min; 45–46% B, 52–53 min; 46–70% B, 53–56 min; 70–80% B, 56–60 min; 80–85% B, 60–62 min; 85–15% B, 62–65 min; flow rate: 0.5 mL/min	Micromass MassLynx-MS, APCI, PI, SIM		[172]
Animal urine*/isoflavones and conjugates		SPE	Eclipse XDB-C8 column (150 mm × 4.6 mm i.d.), A: 0.1% AcOH/H_2O; B: 25% ACN/MeOH; gradient: 8–25% B, 0–40 min; 25–55% B, 40–70 min; 55–100% B, 70–75 min; flow rate: 1 mL/min	Bruker model Esquire-LC multiple IT-MS, ion source not indicated, PI or NI, UV-DAD: 265 and nm		[173]
In vitro biological sample/B-A, F and metabolites		Extraction with ethyl ether	YMCA AM (50 mm × 4 mm i.d., 3 μm) with a 20 mm × 4 mm i.d. guard column, A: MeOH/ACN/H_2O/1 M $AcONH_4$ (1:3:15:1); B: MeOH/ACN/H_2O/1 M $AcONH_4$ (4:10:5:1); gradient: 0–75% B, 0–25 min; flow rate: 1 mL/min	Micromass Quattro Ultima TQ-MS, ESI, PI (identification) ED-CEAD: 7 chanels (quantification)		[174]
Animal urine/deuterated ENL	G	Enzymatic hydrolysis, extraction with ethyl acetate	Hypersil BDS C_{18} column (150 mm × 2.1 mm i.d., 5 μm), A: 5% ACN/0.1% AcOH; B: 40% ACN/0.1% AcOH; gradient: 0–100% B, 0–3 min; held 100% B for 12 min; flow rate: 0.2 mL/min	TQ-MS (API2000), ESI, NI, MRM		[175]

Bovine milk*/D, G, EQ, F, B-A, ENL, END and C		Enzymatic hydrolysis, SPE (C-18), SPE (SiOH)	Nucleosil C_{18} AB column (50 mm × 2.1 mm i.d., 5 μm) equipped with guard column (10 mm × 2.1 mm i.d.), A: MeOH; B: 0.5% AcOH/H_2O; gradient: 70–0% B, 0–12 min; held 0% B for 2 min; flow rate: 0.3 mL/min	Micromass QuattroLC TQ-MS, ESI, PI or NI, MRM		[176]
Human plasma and urine/D, G, EQ, *O*-DMA, ENL, END, MAT and C	Deuterated standards of corresponding analytes and hexestrol for C	Enzymatic hydrolysis, SPE	Prism RP-column (50 mm × 3 mm i.d., 5 μm) equipped with guard column, mobil phase consisting $AcONH_4$, MeOH and ACN in gradient; flow rate: 0.3 mL/min	PE Sciex API III TQ-MS, APCI, NI, MRM	Recovery: 80.7–103.7%, CV: <10%, LOD: 0.1–10.0 ng/mL	[177]
Human urine and serum/D, G, EQ, *O*-DMA, C, ENL, END and MAT	Dueterated standards of corresponding analytes	Enzymatic hydrolysis, SPE	RP-HPLC column (50 mm Prism column), mobile phase not indicated	PE Sciex API III tandem MS, APCI, PI, MRM		[178]
Plant organism/G, quercetin and kaemferol		Extraction with acetone and acetone:MeOH (1:1) (for conjugates) acidic hydrolysis, extraction with ehtyl acetate (for aglycones)	ODS2 RP-column (250 mm × 4.6 mm i.d., 5 μm), A: 1% H_3PO_4 (UV) or 0.1% AcOH (MS)/H_2O; B: ACN; gradient (1): held 5% B for first 5 min, 5–10% B, 5–10 min; 5–15% B, 5–15 min; held 15% B for 5 min; 15–17% B, 20–25 min; 17–23% B, 25–30 min; 23–50% B, 30–65 min (for conjugates), gradient (2): held 27% B for first 5 min, 27–30% B, 5–45 min; 30–100% B, 45–46 min (for aglycones); flow rate: 1.0 mL/min	Bruker Esquire IT-MS; ESI, PI		[179]
In vitro biological sample*/chloro and nitro metabolites of isoflavones			C-8 RP-column (100 mm × 4.6 mm i.d.), A: 10 mM $AcONH_4$/H_2O; B: ACN; gradient: 0–50% B, 0–10 min, held 50% B for 2 min; 50–90% B, 12–15 min; held 90% for 2 min; flow rate: 1.0 mL/min	PE Sciex API III tandem MS, ESI, NI,		[180]
Kudzu dietary supplements/isoflavones	Fluoresein	Extracted with aqueous EeOH (80%)	C-8 RP-column (220 mm × 4.6 mm i.d.) equipped with guard column, A: 10% ACN/0.1% TFA; B: 90% ACN/0.1% TFA; gradient: 0–70% B, 0–30 min; flow rate: 1.5 mL/min (quantitation by UV), C-8 RP-column (150 mm × 4.6 mm i.d.), A: 10 mM $AcONH_4$/H_2O; B: 10 mM $AcONH_4$/ACN; gradient: 0–40% B, 0–30 min; flow rate: 0.1 mL/min	PE Sciex API III MS, ESI, NI, micromass Q-TOF-MS/MS, ESI, PI or NI, UV-DAD: 262 (quantitation)		[181]

IT-MS, ion trap mass spectrometer; TQ-MS, triple quadrupole mass spectrometer; ESI, electrospray ionization; APCI, atmospheric pressure chemical ionization; PI, positive ion source; NI, negative ion source; SIM, selected ion monitoring; MRM, multiple reaction monitoring; SRM, selected reaction monitoring; SPE, solid-phase extraction; R.S.D., relative standard derivation; CV, coefficient variation; LOQ, limit of quantitation; LOD, limit of detection; TFA, trifluoroacidic acid; CAN, acetonitrile. *Analyte*: D, daidzein; G, genistein; GL, glycitein; F, formononetin; C, coumestrol; B-A, biochanin-A; EQ, equol; *O*-DMA, *O*-desmethylangolensin; ENL, enterolactone; END, elactrodiol; MAT, matairesinol; SECO, secoisolariciresinol, BPA, bisphenol A; NP, nonylphenol.

* Qualitative identification. The ratio of mobile phase components is in v/v.

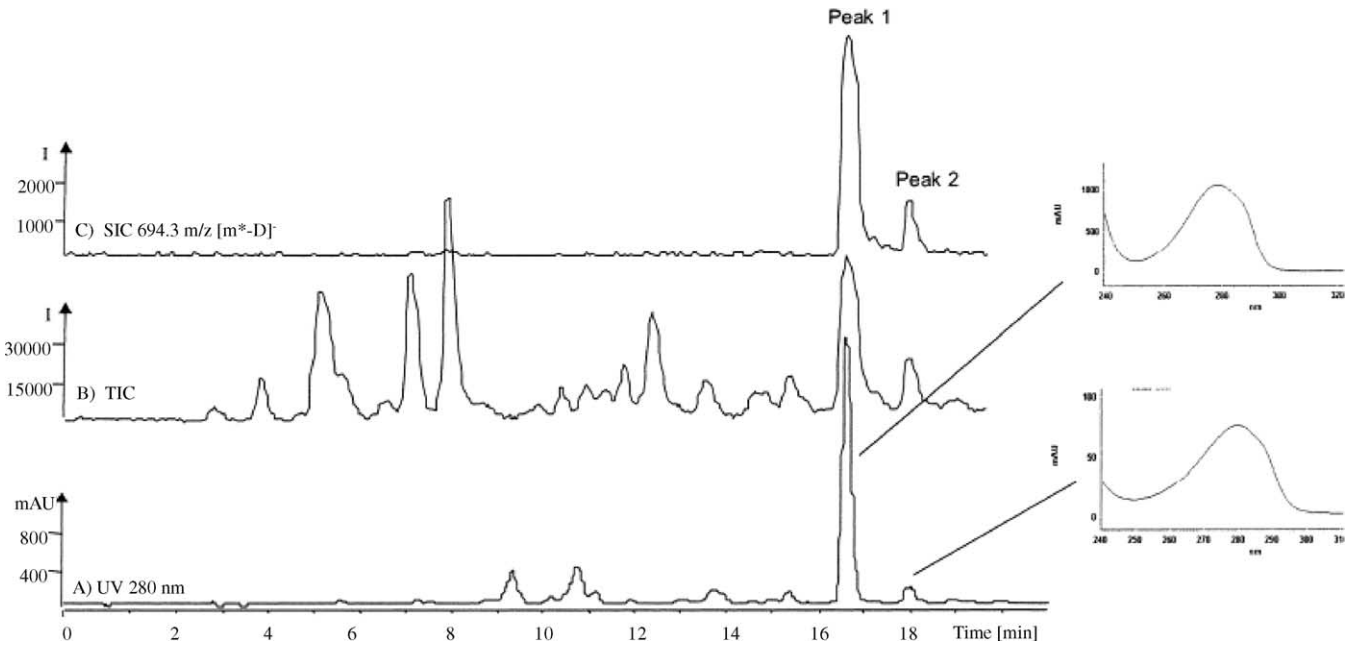


Fig. 12. On-line LC–NMR–MS analysis of a flaxseed extract: (A) UV spectrum and chromatogram at 280 nm; UV spectra (240–340 nm) of peaks 1 and 2 are superimposed. (B) Total ion chromatogram (TIC). (C) Selected ion chromatogram (SIC) of *m/z* 694.3. Peaks 1 and 2 indicate the two diastereoisomers of secoisolariciresinol diglucoside (from [191], with the permission from Elsevier Science).

time, capillary electrophoresis has been widely applied to analysis of phytoestrogens in plants, plant products and biological matrices as an alternative technique over the last decade. CE separation is based on the differences in electrophoretic mobility of charged analytes in an electric field in small diameter capillaries. Till today, several CE techniques have been developed including the most commonly used capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MEKC). Since most of the phytoestrogens and their metabolites contain phenolic hydroxyl group and shows weak acidic nature, the utilization of CZE and MEKC methods are generally performed based on a borate run buffer at alkaline pH to ensure the analytes are charged for electrophoretic separation. As with HPLC, most of the detection, e.g. UV, laser-induced fluorescence (LIF), ED, MS and NMR can be coupled to monitor CE separation. Previous reviews have summarized the applications of CE method for analysis of phytoestrogens and other natural phenolics [8,195,196].

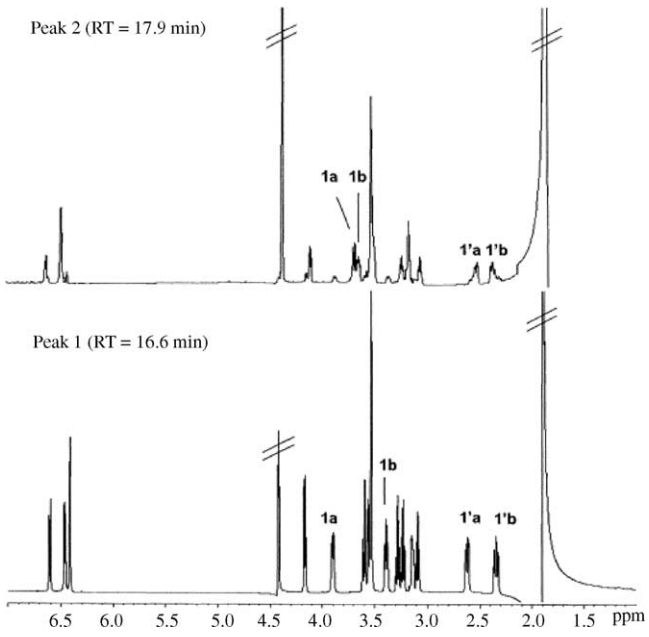


Fig. 13. On-line ^{1}H NMR (600 MHz) spectra of the two diastereoisomers of secoisolariciresinol diglucoside (from [191], with the permission from Elsevier Science).

2.3.1. Capillary zone electrophoresis

Capillary zone electrophoresis is the most widely used type of CE because of its simplicity and versatility. Separation occurs as charged analytes are propelled through the capillary at different velocities depending on size and charge, whereas the neutral species migrate at the same speed. CZE is usually performed with electrical potentials of up to 30 kV. Separation efficiencies as high as 500,000 theoretical plates can be obtained for low molecular weight compounds in 30 min using such potentials [197,198]. Aussenac et al. [199] presented a CZE method combined with multiwavelength UV detector (260 nm) for analysis of isoflavones in soybean matrices extracted at different temperature and with different solvent components. In that study, the electrokinetic separation was performed on fused silica capillary column (67 cm × 50 μm i.d.) at the electrical field strength of 23.5 kV using the running buffer of either 30 mM NaH_2PO_4 (pH

9.5–10.5) or 50–100 mM H_3BO_3 at pH 10.5. Nine flavonoids in grape wine including quercetin, kaempferol and apigenin were quantified by means of CZE coupled with UV detector (250 nm), as well as HPLC/UV-DAD [200]. SPE (C-18 cartridge) was used in sample preparation. The optimum separation condition of CZE was with 35 mM borax (pH 8.9), and applied 240 V/cm electrical field strength on a fused sillica capillary of 70 cm (effective length: 45 cm) × 75 μm i.d. For all analytes, the achieved recovery ranged from 90.1 to 99.8% and the relative standard deviations (R.S.D.) were under 5.7%. Vaher and Koel [201] have investigated the polyphenols including *trans*-resveratral, quercetin and catechin in various parts of Japanese knotweed. CZE method with UV detector (240 nm) was developed for the separation of polyphenolic compounds using both water based borate buffer and nonaqueous media (nonaqueous CE, NACE). Experiments were conducted using an uncoated capillary (75 cm × 50 μm i.d.) with electrical field strength of 18 kV. Dabas et al. reported [202] recently on a method to optimize the separation conditions of soy isoflavones in the urine of subjects who consumed large amounts of soy and soy-derived products by CZE. In their study, biological matrices were treated using SPE for sample prepurification. Due to the phenolic nature of isoflavones, pHs (8.20–9.90) of running buffer were tested.

Electrochemical detection was also applied to monitoring the CE separation for phytoestrogen. Gao et al. [203] developed a CE–ED method for determination of *trans*-resveratrol in wine, Chinese herbal medicine *Polygonum cuspidatum* and herbal preparation. CE separation was performed on a fused sillica capillary of 65 cm × 25 μm i.d. using the running buffer of 100 mM H_3BO_3–$Na_2B_4O_7$ (pH 9.24), and 10 kV electrical field strength was applied. ED was employed to monitor the analyte at detection potential +850 mV. Limit of detection was found to be 59.6 ng/mL. A similar CE–ED method was also reported for analysis of flavonoids in *Hippophae rhamnoides* by this laboratory [204]. A 75 cm × 25 μm i.d. fused sillica capillary was used for the separation with the running buffer of 40–60 mM H_3BO_3–$Na_2B_4O_7$ (pH 9.0) at electrical field strength of 14 kV. ED potential was maintained at +950 mV. For all analytes, limit of detection ranged from 130 to 590 ng/mL.

2.3.2. Micellar electrokinetic capillary chromatography

In CZE technique, the separation is carried out in free solution. The chemistry of analytes is not particularly important, only their migration properties. Micellar electrokinetic capillary chromatography (MEKC), in comparison, can create a pseudo-stationary phase in the capillary by adding micelles of surfactants such as sodium dodecyl sulfate (SDS), allowing for the separation based on the molecular properties. This is especially useful for the separation of neutral molecules. Baggett et al. [205] described the development of MEKC method with UV-PDA for the analysis of isoflavones in comparison to HPLC. Different fused sillica capillaries (50 and 75 μm i.d.) were applied to CE separation. The role of several factors such as electrical field strength, buffer composition and buffer pH were optimized to ensure the selectivity of analytes in complex plant samples. The addition of 1,2-hexanediol to SDS electrolyte provided the improved resolution of adjacent analytes. A MEKC method coupled with UV-DAD (280 nm) was established for determination of flavonoids in the flowers of *Paulomnia tomentosa* by Jiang et al. [206]. CE separation was carried out on an untreated fused sillica capillary of 30 cm effective length (50 μm i.d.) at electrical field strength of 15 kV. The effects of primary experimental conditions were assessed and the optimum factors of a 20 mM sodium borate buffer (pH 10.0), 10 mM SDS and 5% MeOH were selected as the most suitable electrolytes for this separation. MEKC was applied to the analysis of natural resveratrol and related synthesized glucoside with UV-DAD (254 nm) by Brandolini et al. [207]. The separation was obtained with a fused sillica capillary (75 cm × 57 μm i.d.) using the running buffer of 20 mM $Na_2B_4O_7$, 25 mM PEG 400 (poly ethylene glycol), and 25 mM SDS with addition of 10% MeOH just before use. The limit of detection of 0.5 ppm was achieved in wine for resveratrol, thus ensuring the desired high efficiency for this method.

2.3.3. Capillary electrochromatography

Capillary electrochromatography (CEC) is a new and exciting hybrid separation technique that couples the high separation efficiency of CZE with HPLC, and employs an electric field rather than hydraulic pressure to propel the mobile phase through a packed bed. Since there is no back-pressure, it is possible to use small diameter packing and thereby achieve very high efficiencies. Starkey et al. [208] described the application of CEC with UV-PDA detector for determination of trace level isoflavone phytoestrogens in blood samples from human volunteers, whose diet was supplemented by a soy-derived product. Separation was performed on a developed C_{12} CEC column (24.5 cm) using the mobile phase of 30% ACN and 50% MeOH in 2.4 mM ammonium format buffer (pH 2.4) at electrical field strength of 800 V/cm, and apigenin was used as the internal standard. With on-column preconcentration, this method enables to detect low-ppb levels of analytes in human plasma.

2.4. Immunoassays

Immunoassay has been utilized widely for analysis of phytoestrogens and metabolites particularly in biological samples because of its numerous advantages of high throughput screening purposes, potential of high sensitivity, specificity and relatively low cost. Immunoassay tests use antibodies that have been specifically developed to bind with a target compound or class of compounds known as antigens. The binding sites on an antibody attach precisely and noncovalently to their corresponding target analyte (antigen). Because binding is based on the antigen's physical shape rather than its chemical properties, antibodies will not respond to substances that have dissimilar structures. In phytoestrogen immunoassay, the first step is to design and

synthesize a suitable hapten, a structurally specific derivative of phytoestrogen, which is used to raise the corresponding antibody. All the phytoestrogens and metabolites comprise at least one hydroxyl group. This can be used for the introduction of an *O*-carboxyalkyl spacer arm to form a suitable hapten. At present, the commonly used immunoassay types for phytoestrogen analyses include radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and time-resolved fluoroimmunoassay (TR-FIA). Previous papers have reviewed the immunoassay applications on phytoestrogen analysis through 2001 [4,8,25].

2.4.1. Radioimmunoassay

Radioimmunoassay (RIA) techniques are based on the reaction between an antibody and an antigen (target analyte). There are several ways to quantify the antigen concentration, but the most frequently used method is the indirect assay. In this assay, a known quantity of radioactively labeled antigen (iodine-125) is mixed with a dilution series of unlabeled antigen. The dilution series is brought to reaction with a fixed amount of antibody specific against the antigen. Since unlabeled and labeled antigens compete with each other for the antibody binding sites, the greater the quantity of unlabeled antigen, the less labeled antigen is bound to the antibody and vice versa. After a fixed time, a second antibody directed against the first antibody is used which leads to the formation of large complexes which upon centrifugation are counted with a radioactive counter. RIA methods are very sensitive and allow to detecting nano- or picomoles of molecules. Lapcik et al. [209] recently established an RIA system for determining coumestrol in a legume plant sample based on the polyclonal antiserum and a ^{125}I-labeled hapten-tyrosine methyl ester conjugate as the radioligand. In that study, the hapten 3-*O*-carboxymethylcoumestrol was prepared by a partial alkylation of coumestrol with ethyl chloroacetate in acetone alkalized with potassium carbonate. 3-*O*-Ethoxycarbonylmethylcoumestrol was purified and was finally hydrolyzed with formic acid, in which 1D and 2D NMR were applied to structure elucidation. The hapten was then conjugated to bovine serum albumin (BSA) and used for immunization. Plant samples were assessed using both RIA developed and LC–MS (under positive ESI mode). Compared to LC–MS, the coumestrol levels obtained by RIA were slightly overestimated (RIA result = 1.18 × HPLC–MS result), and there was good correlation (r^2 = 0.8989) between both methods.

2.4.2. Enzyme-linked immunosorbent assays

Because of the requirement to use radioactive substances, RIAs are currently being replaced by other immunologic assays. Enzyme-linked immunosorbent assays (ELISA) are highly sensitive and more specific compared favorably to RIAs, and also these techniques have the added advantages of not needing radioisotopes or a radiation-counting apparatus. The basic principle of an ELISA is to use an enzyme to detect the binding of antigen and antibody. The enzyme converts a colorless substrate (chromogen) to a colored product, indicating the presence of antigen-antibody binding. The concentration of the target analyte can be determined by observing the color change by using a photometer or spectrophotometer to measure the precise change in the color of the reaction. An ELISA can be used to detect either the presence of antigens or antibodies in a sample, depending on how the test is designed. Bennetau-Pelissero et al. [210] reported the synthesis of seven carboxylic acid haptens of four common phytoestrogen isoflavones formononetin, daidzein, biochanin A and genistein, and daidzein metabolite equol, with the spacer arm on the oxygen atom at the C7 position for formononetin, daidzein, equol, biochanin A and genistein, and at the C8 position for formononetin and daidzein only. The different haptens were coupled to BSA and to swine thyroglobulin (Thyr), and ELISAs were then performed based on competition between free phytoestrogens and the Thyr-hapten conjugates for specific antibodies. Also the synthesis of novel hapten-protein conjugates (hapten-BSA and heptan-Thyr) for production of highly specific antibodies to daidzein, genistein and fomornonetin were reported by this laboratory [211]. They then applied that ELISA approach and were able to measure two major soy isoflavones daidzein and genistein in food and human fluid [212].

2.4.3. Time-resolved fluoroimmunoassay

Lanthanide, especially europium (Eu) and samarium (Sm), can form highly fluorescent chelates, which have very long fluorescence decay times and a large Stokes' shift. Time-resolved fluoroimmunoassay (TR-FIA) is the technique based on these unique fluorescence properties of the lanthanide chelates, in which Eu-labeled antibodies are used as the most common application. This approach allows sensitive and specific detection of lanthanide chelates by eliminating or reducing non-specific fluorescence by pulsing excitation light. Because the lanthanide chelates can be measured as sensitively as radioisotopes and are stable and easy to handle, they are widely used as tracers for analysis of phytoestrogen and metabolites in the biological matrices, particularly for measuring unconjugated phytoestrogens in plasma and tissue samples. The application of this approach achieves the advantages of reagent stability and lack of radiation over RIA. Additionally, it can also provide a 10- to 100-fold increase in sensitivity and assay range compared to ELISA and fluoroimmunoassay (FIA) methods. L'homme et al. [213] presented a TR-FIA method for determining *O*-desmethylangolensin (*O*-DMA), a metabolite of phytoestrogen daidzein in plasma and urine using a europium chelate as a label. Synthesized 4″-*O*-carboxymethyl-*O*-DMA coupled to BSA was used as the antigen in immunization. Europium chelate tracers were also synthesized using the same 4″-*O*-derivative of the —methyldeoxybenzoin. Sample preparation involved enzymatic hydrolysis and ether extraction prior to TR-FIA. Antisera cross reactivity was tested with the common isoflavone metabolites in human, including angolensin, dihydrogenistein, dihydrodaidzein, equol, 6′-OH-

angolensin, *trans*-4-OH-equol, 6′-OH-*O*-DMA, *cis*-4-OH-equol and 5-OH-equol. Results from this TR-FIA method were confirmed by GC–MS analysis, and good correlation coefficients were achieved. Experimental conditions were optimized and methodological parameters, e.g. accuracy, precision and sensitivity were assayed. This research group was latter described a similar TR-FIA approach for analysis of daidzein metabolite equol in human plasma and urine [214]. Another TR-FIA for the determination of mammalian lignan enterolactone in animal urine was also reported by this same laboratory [95].

2.5. Sample preparation

Phytoestrogen containing samples can be divided into two categories: (1) plant origin or derived diets, such as soybean, flaxseed and their produced products and (2) biological matrices (e.g. human or animal urine, serum, tissue and in vitro cultures). Sample preparation procedures for this first category is relatively simple, and generally involves direct solvent extraction with aqueous MeOH/EtOH for glycoside conjugate containing samples [31,96,111,113,119–121,123,137,167–169,172,181], or with ether and/or ethyl acetate for aglycone only containing samples [67,93,103]. Enzymatic and/or acidic hydrolysis during extraction is sometimes employed depending on the study purpose, when only isoflavone and lignan aglycones are examined [31,67,93,103,111,114,115,125,137,139,167]. Flaxseed is among the most lignan enriched plant sources and contains large amount of lipids and other non-polar components, which can interfere with the analysis and shorten column life. Thus, partitioning with *n*-hexane prior to extraction is often applied for analysis of flaxseed and/or its derived products [67,96,119,139].

Ingested isoflavones and lignans together with their metabolites in biological matrices are mostly present as glucuronic acid or sulfate conjugations. Thus, enzymatic hydrolysis of these conjugates using a mixed β-glucuronidase and sulphatase preparation is generally employed to release the aglycone form [102,107,109,112,117,138,160,161,166]. The common source of these enzymes is from *H. pomatia*. Complex biological matrices comprise a variety of impurities, such as proteins and lipids. Following hydrolysis, preconcentration of the analytes from the aqueous buffered reaction medium involved in either solid-phase extraction (SPE, using C_{18} Sep-Pak cartridges) [109,110,140,160,161,164–166,177,178], or solvent extraction with either diethyl ether or ethyl acetate [66,162,175] is performed or a combination of solid-phase and organic solvent extraction is used [85,92,94,112]. Sometimes SPE treatment is applied prior to enzymatic hydrolysis [85,92,94,110,160], and other chromatographic methods using Sehphadex-LH20 for biological sample pre-purification are also used [85,94,109,110,138], as well as ion-exchanged chromatography [92,102,138]. As phytoestrogen precursors and their metabolites contain polar hydroxyl and/or carboxyl groups, derivatization is needed to increase the volatility of each analyte prior to GC–MS analysis. The common reagents are *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA–TMCS) and produce the derivatives of trimethylsilyl ether (TMS). Sample silylation with pyridine–hexamethyldisilazane–trimethylchlorosilane (Py–HMDS–TMCS) (9:3:1, v/v/v) is sometimes employed [85].

Due to the multiple steps in sample preparation, such as organic solvent extraction and solid-phase extraction (SPE, C-18, Shephadex-LH20 or ion-exchange chromatography) in sample pre-purification, hydrolysis of conjugates (β-glucuronidase and/or sultfatase) and derivatization to increase the volatility, any unknown losses during the processing are totally uninvited. To adjust any such looses, internal standards are generally added during sample preparation to correct for losses. These standards usually are stable isotopic labels such as deuterated (^{2}H) or carbon-13 (^{13}C) labeled forms of the corresponding phytoestrogens and their metabolites (for MS detection only) [92,93,160,161], or chemical analogous with similar chemical structure and properties that are not naturally present in the sample to be studied [109,111,112,115,159,162]. The sample preparation procedures for the applications of GC–MS and HPLC based analysis are summarized in Tables 1–3.

3. Conclusions

Phytoestrogenic compounds, predominantly phenolic isoflavones and lignans, have been shown to induce a wide range of hormone-related activities, with effects associated with breast and prostate cancer, cardiovascular disease and conditions associated with bone health and menopause. Driven largely by scientific research on these potential health benefits, a variety of analytical methods for analysis of these phytoestrogens and metabolites in plants and their derived products and biological matrices have been established using GC–MS, HPLC and CE based methodology and immunoassays. GC combined with MS provides reliable and conclusive analytical information for the identification and quantitation of phytoesrogens and metabolites, particularly in biological samples. Because of the high chromatographic resolution, specificity and good sensitivity, as well as the achieved capability of structure elucidation, this technique had predominated the application of phytoestrogen pharmacokinetic study prior to the employment of LC–MS. However, the laborious sample preparation procedures, e.g. pre-purification, fractionation, hydrolysis and derivatisation of samples limited its application.

HPLC is presently the most widespread chromatographic technique in the investigation of these compounds, due to its compatibility to various detections, e.g. UV, ED, fluorescence detection, MS and NMR. This approach is easy and suitable to separate the polar hydroxyl and/or carboxyl group consisting phytoestrogens on reversed phase column, yet in general achieves poor chromatographic resolution. All the

phenolic phytoestrogens have at least one aromatic ring with the maximum UV absorption ranged from 230 to 280 nm, so UV or multiwavelenth UV detector has been found to be very useful. However, UV detection has a disadvantage in low sensitivity and is generally applied to examining the relatively high concentration phytoestrogens in plant or plant derived samples. Fluorescence detection and electrochemical detection rather than UV detection has been used to increase the sensitivity. Compared to GC–MS, HPLC coupling with MS detector (LC–MS) has the advantages of higher precision, less manipulation, and applicability to non-volatile analytes with direct injection of the liquid samples. The application of LC–MS is a fast moving field, and over last decade many new and interesting techniques have been developed including different MS analyzers such as single quadrupole, triple quadrupole, ion trap and Q-TOF mass spectrometers equipped with ESI and/or APCI interfaces. LC–MS analysis, which is both sensitive and requires relatively simple sample pretreatment, is particularly suitable for determining the phytoestrogens and metabolites in biological matrices. These techniques have presently been the majority of analytical approaches for absorption, distribution, metabolism and excretion (ADME) bioanalyses on new drug discovery, and large amount of literatures has been published in this area. HPLC combined with UV (multiwavelength), MS or tandem MS provides a great deal of preliminary information for the content and nature of phytoestrogens in a crude sample. While that information is helpful and may be sufficient for known compound identification in complex mixtures, it remains insufficient to elucidate the structure of an unknown constituent and cannot distinct the isomers, especially the geometrical and optical isomers. The dihyphenated LC–NMR system allows to providing precious complementary structure information and can resolve this latter problem. Presently NMR remains a rather insensitive detection, only proton NMR spectra can be achieved.

Capillary electrophoresis is a relatively new analytical technique and has the advantages of high separated resolution (efficiencies in the order of GC) and low amount of sample required. Similar to HPLC, this device can be adapted to different detections, e.g. UV, ED, fluorescence detection, MS and NMR. Non-chromatographic immunoassay is one of the most sensitive methods. Because of high throughput screening and relatively low cost per sample, it is particularly suitable for large amount of sample assays. Disadvantage of this approach is the possible cross-reactivity over the similar compounds and overestimation of analytes could be occurred. Additionally, preparing the antibodies for corresponding analytes remains a challenge issue.

Acknowledgments

This work was supported in part by NIH grant OD-00-004, and the New Use Agriculture and Natural Plant Products Program and was conducted as part of the National Botanical Center for Age-Related Diseases. Authors also acknowledge the support of the New Jersey Agricultural Station and Cook College, Rutgers University and the New Jersey Farm Bureau, Trenton, NJ.

References

[1] S. Hendrich, J. Chromatogr. B 777 (2002) 203.
[2] H. Adlercreutz, Baillieres Clin. Endocrinol. Metab. 12 (1998) 605.
[3] R.S.R. Zand, D.J.A. Jenkins, E.P. Diamandis, J. Chromatogr. B 777 (2002) 219.
[4] A.A. Hoikkala, E. Schiavoni, K. Wahala, Br. J. Nutr. 89 (2003) 5.
[5] W.M. Mazur, J.A. Duke, K. Wähälä, S. Rasku, H. Adlercreutz, J. Nutr. Biochem. 9 (1998) 193.
[6] A.F.M. Kardinaal, M.S. Morton, Eur. J. Clin. Nutr. 52 (1998) 850.
[7] H. Adlercreutz, W. Mazur, K. Stumpf, A. Kilkkinen, P. Pietinen, K. Hulten, G. Hallmans, Biofactors 12 (2000) 89.
[8] C.C. Wang, J.K. Prasain, S. Barnes, J. Chromatogr. B 777 (2002) 3.
[9] L. Wang, J. Chromatogr. B 777 (2002) 289.
[10] E. Farmakalidis, P.A. Murphy, J. Agric. Food Chem. 33 (1985) 385.
[11] A. Cassidy, M. Faughnan, Proc. Nutr. Soc. 59 (2000) 489.
[12] J. Bowe, X.F. Li, D. Sugden, J.A. Katzenellenbogen, B.S. Katzenellenbogen, K.T. O'Byrne, J. Neuroendocrinol. 15 (2003) 105.
[13] T.A. Grese, H.W. Cole, D.E. Magee, D.L. Phillips, P.K. Shetler, L.L. Short, A.L. Glasebrook, H.U. Bryant, Bioorg. Med. Chem. Lett. 6 (1996) 2683.
[14] L.A. Henry, D.M. Witt, Horm. Behav. 41 (2002) 220.
[15] D. Bagchi, H.G. Preuss, M. Bagchi, S.J. Stohs, Res. Commun. Pharmacol. Toxicol. 5 (2000) 107.
[16] P. Cos, T. De Bruyne, S. Apers, B.D. Vanden, L. Pieters, A.J. Vlietinck, Planta Med. 69 (2003) 589.
[17] M.J. De Kleijn, Y.T. Van Der Schouw, P.W. Wilson, H. Adlercreutz, W. Mazur, D.E. Grobbee, P.F. Jacques, J. Nutr. 131 (2001) 1826.
[18] W. Wuttke, H. Jarry, T. Becker, A. Schultens, V. Christoffel, C. Gorkow, D. Seidlova-Wuttke, Maturitas 44 (2003) 9S.
[19] H. Adlercreutz, Mol. Biol. 83 (2002) 113.
[20] K.D.R. Setchell, A. Cassidy, J. Nutr. 129 (1999) 758S.
[21] H. Adlercreutz, Lancet Oncol. 3 (2002) 364.
[22] D.M. Tham, C.D. Gardner, W.L. Haskell, J. Clin. Endocrinol. Metab. 83 (1998) 2223.
[23] H. Adlercreutz, W. Mazur, Ann. Med. 29 (1997) 95.
[24] D.C. Knight, J.A. Eden, Obstet. Gynecol. 87 (1996) 897.
[25] B. Raffaelli, A. Hoikkala, E. Leppala, K. Wahala, J. Chromatogr. B 777 (2002) 29.
[26] J.W. Lampe, J. Nutr. 133 (2003) 956S.
[27] M.A. Mendez, M.S. Anthony, L. Arab, J. Nutr. 132 (2002) 2127.
[28] R.A. Dixon, D. Ferreira, Phytochemistry 60 (2002) 205.
[29] M.J. Messina, Am. J. Clin. Nutr. 70 (1999) 439S.
[30] M. Messina, Eur. J. Cancer 36 (2000) 71S.
[31] Q. Wu, M. Wang, J.E. Simon, J. Chromatogr. A 1016 (2003) 195.
[32] L.Z. Lin, X.G. He, M. Lindenmaier, J. Yang, M. Cleary, S.X. Qiu, G.A. Cordell, J. Agric. Food Chem. 48 (2000) 354.
[33] X.G. He, L.Z. Lin, L.Z. Lian, J. Chromatogr. A 755 (1996) 127.
[34] E.D. Rijke, A. Zafra-Gómez, F. Ariese, U.A.T. Brinkman, C. Gooijer, J. Chromatogr. A 932 (2001) 55.
[35] W. Oleszek, A. Stochmal, Phytochemistry 61 (2002) 165.
[36] H.Y. Kim, J.H. Hong, D.S. Kim, K.J. Kang, S.B. Han, E.J. Lee, H.W. Chung, K.H. Song, K.A. Sho, S.J. Kwack, S.S. Kim, K.L. Park, S.K. Lee, M.C. Kim, C.M. Kim, I.S. Song, Food Sci. Biotech. 12 (2003) 29.
[37] S.M. Boue, T.E. Wiese, S. Nehls, M.E. Burow, S. Elliott, C.H. Carter-Wientjes, B.Y. Shih, J.A. McLachlan, T.E. Cleveland, Agric. Food Chem. 51 (2003) 2193.

[38] T. Nomura, T. Fukai, T. Akiyama, Pure Appl. Chem. 74 (2002) 1199.
[39] X. Ma, P. Tu, Y. Chen, T. Zhang, Y. Wei, Y. Ito, J. Chromatogr. A 992 (2003) 193.
[40] Y. Tang, F. Lou, J. Wang, S. Zhuang, J. Nat. Prod. 64 (2001) 1107.
[41] H. Yamamoto, P. Zhao, K. Inoue, Phytochemistry 60 (2002) 263.
[42] K.R. Markham, W.T. Swift, T.J. Mabry, J. Org. Chem. 33 (1968) 462.
[43] W.A. Asomaning, E. Otoo, O. Akoto, I.V. Oppong, I. Addae-Mensah, R. Waibel, H. Achenbach, Phytochemistry 51 (1999) 937.
[44] C.N. Manoj, R.P. Rao, M.G. Sethuraman, N.S. Nagarajan, M. Kaliaperumal, Magn. Reson. Chem. 41 (2003) 227.
[45] P. Bednarek, R. Franski, L. Kerhoas, J. Einhorn, P. Wojtaszek, M. Stobiecki, Phytochemistry 56 (2001) 77.
[46] N.C. Veitch, P.S.E. Sutton, G.C. Kite, J. Nat. Prod. 66 (2003) 210.
[47] M.K. Tsanuo, A. Hassanali, A.M. Hooper, Z. Khan, F. Kaberia, J.A. Pickett, L.J. Wadhams, Phytochemistry 64 (2003) 265.
[48] V. Rukachaisirikul, Y. Sukpondma, C. Jansakul, W.C. Taylorm, Phytochemistry 60 (2002) 827.
[49] A.I. Calderon, C. Terreaux, K. Schenk, P. Pattison, J.E. Burdette, J.M. Pezzuto, M.P. Gupta, K. Hostettmann, J. Nat. Prod. 65 (2002) 1749.
[50] I. Orhan, S. Nasim, B. Sener, F. Ayanoglu, M. Ozguven, M.I. Choudhary, R. Attaur, Phytother. Res. 17 (2003) 575.
[51] R. Tsao, R. Yang, J.C. Young, J. Agric. Food Chem. 51 (2003) 6445.
[52] A. Degenhardt, S. Habben, P. Winterhalter, J. Chromatogr. A 943 (2002) 299.
[53] J.D. Ford, K.S. Huang, H.B. Wang, L.B. Davin, N.G. Lewis, J. Nat. Prod. 64 (2001) 1388.
[54] L.U. Thompson, P. Robb, M. Serraino, F. Cheung, Nutr. Cancer 16 (1991) 43.
[55] R.D. Harworth, Nature (Lond.) 147 (1941) 255.
[56] I.R. Rowland, H. Wiseman, T.A.B. Sanders, H. Adlercreutz, E.A. Bowey, Nutr. Cancer 36 (2000) 27.
[57] P.D. Nesbitt, Y. Lam, L.U. Thompson, Am. J. Clin. Nutr. 69 (1999) 549.
[58] M.S. Morton, G. Wilcox, M.L. Wahlqvist, K. Griffiths, J. Endocrinol. 142 (1994) 251.
[59] H. Adlercreutz, T. Fotsis, M.S. Kurzer, K. Wähälä, T. Mäkelä, T. Hase, Anal. Biochem. 225 (1995) 101.
[60] J. Liggins, R. Grimwood, S.A. Bingham, Anal. Biochem. 287 (2000) 102.
[61] H. Adlercreutz, J. Van Der Wildt, J. Kinzel, H. Attalla, K. Wahala, T. Makela, T. Hase, T. Fotsis, J. Steroid Biochem. Mol. Biol. 52 (1995) 97.
[62] S.P. Borriello, K.D.R. Setchell, M. Axelson, A.M. Lawson, J. Appl. Bacteriol. 58 (1985) 37.
[63] L.P. Meagher, G.R. Beecher, J. Food Compos. Anal. 13 (2000) 935.
[64] K. Kobashi, T. Akao, M. Hattori, T. Namba, Bifidobact. Microflora 11 (1992) 9.
[65] P. Johnsson, A. Kamal-Eldin, L.N. Lundgren, P. Aman, J. Agric. Food Chem. 48 (2000) 5216.
[66] S. Heinonen, T. Nurmi, K. Liukkonen, K. Poutanen, K. Wähälä, T. Deyama, S. Nishibe, H. Adlercreutz, J. Agric. Food Chem. 49 (2001) 3178.
[67] T. Sicilia, H.B. Niemeyer, D.M. Honig, M. Metzler, J. Agric. Food Chem. 51 (2003) 1181.
[68] S. Milligan, J. Kalita, V. Pocock, A. Heyerick, L. De Cooman, H. Rong, D. De Keukeleire, Reproduction (Camb., UK) 123 (2002) 235.
[69] O. Schaefer, M. Huempel, K.H. Fritzemeier, R. Bohlmann, W.D. Schleuning, J. Steroid Biochem. Mol. Biol. 84 (2003) 359.
[70] B.E. Knuckles, D. DeFremery, G.O. Kohler, J. Agric. Food Chem. 24 (1976) 1177.
[71] L.W. Sumner, N.L. Paiva, R.A. Dixon, P.W. Geno, J. Mass Spectrom. 31 (1996) 472.
[72] R. Edwards, S.A. Tiller, A.D. Parry, J. Plant Physiol. 150 (1997) 603.
[73] K.D.R. Setchell, N.M. Brown, E. Lydeking-Olsen, J. Nutr. 132 (2002) 3577.
[74] G.E. Joannou, G.E. Kelly, A.Y. Reeder, M. Waring, C. Nelson, J. Steroid Biochem. Mol. Biol. 54 (1995) 167.
[75] S. Heinonen, K. Wahala, H. Adlercreutz, Anal. Biochem. 274 (1999) 211.
[76] S.E. Kulling, L. Lehmann, M. Metzler, J. Chromatogr. B 777 (2002) 211.
[77] S.E. Kulling, D.M. Honig, T.J. Simat, M. Metzler, J. Agric. Food Chem. 48 (2000) 4963.
[78] S.E. Kulling, D.M. Honig, M. Metzler, J. Agric. Food Chem. 49 (2001) 3024.
[79] K.D.R. Setchell, A.M. Lawson, S.P. Harkness, H. Gordon, D.M.L. Morgan, D.N. Kirk, H. Adlercreutz, L.C. Anderson, M. Axelson, Lancet 2 (1981) 4.
[80] K.D.R. Setchell, A.M. Lawson, E. Conway, N.F. Taylor, D.N. Kirk, G. Cooley, R.D. Farrant, S. Wynn, M. Axelson, Biochem. J. 197 (1981) 447.
[81] C. Bannwart, H. Adlercreutz, K. Wahala, G. Brunow, T. Hase, Clin. Chim. Acta 180 (1989) 293.
[82] R.S. Gohlke, Anal. Chem. 31 (1959) 535.
[83] C. Brunee, L. Jenckel, K. Kronenberger, Z. Anal. Chem. 197 (1963) 42.
[84] R. Ryhage, Anal. Chem. 36 (1964) 759.
[85] S. Heinoxxnen, K. Wahxxala, H. Adlercrxxeutz, Anal. Biochem. 274 (1999) 211.
[86] K. Wahala, S. Rasku, K. Parikka, J. Chromatogr. B 777 (2002) 111.
[87] K.D. Setchell, A.M. Lawson, F.L. Mitchell, H. Adlercreutz, D.N. Kirk, M. Axelson, Nature 287 (1980) 740.
[88] T. Fotsis, R. Heikkinen, H. Adlercreutz, M. Axelson, K.D. Setchell, Clin. Chim. Acta 121 (1982) 361.
[89] H. Adlercreutz, T. Fotsis, R. Heikkinen, J.T. Dwyer, M. Woods, B.R. Goldin, S.L. Gorbach, Lancet 2 (1982) 1295.
[90] H. Adlercreutz, P.I. Musey, T. Fotsis, C. Bannwart, K. Wahala, T. Makela, G. Brunow, T. Hase, Clin. Chim. Acta 158 (1986) 147.
[91] H. Adlercreutz, T. Fotsis, C. Bannwart, K. Wahala, T. Makela, G. Brunow, T. Hase, J. Steroid Biochem. 25 (1986) 791.
[92] H. Adlercreutz, T. Fotsis, C. Bannwart, K. Wahala, G. Brunow, T. Hase, Clin. Chim. Acta 199 (1991) 263.
[93] W. Mazur, T. Fotsis, K. Wahala, S. Ojala, A. Salakka, H. Adlercreutz, Anal. Biochem. 233 (1996) 169.
[94] S.M. Heinonen, A. Hoikkala, K. Wahala, H. Adlercreutz, J. Steroid Biochem. Mol. Biol. 87 (2003) 285.
[95] C. Nicolle, C. Manach, C. Morand, W. Mazur, H. Adlercreutz, C. Remesy, A. Scalbert, J. Agric. Food Chem. 50 (2002) 6222.
[96] L.P. Meagher, G.R. Beecher, V.P. Flanagan, B.W. Li, J. Agric. Food Chem. 47 (1999) 3173.
[97] H. Adlercreutz, T. Fotsis, J. Lampe, K. Wahala, T. Makela, G. Brunow, T. Hase, Scand. J. Clin. Lab. Invest. Suppl. 215 (1993) 5.
[98] H. Adlercreutz, T. Fotsis, S. Watanabe, J. Lampe, K. Wahala, T. Makela, T. Hase, Cancer Detect. Prev. 18 (1994) 259.
[99] M. Uehara, O. Lapcik, R. Hampl, N. Al-Maharik, T. Makela, K. Wahala, H. Mikola, H. Adlercreutz, J. Steroid Biochem. Mol. Biol. 72 (2000) 273.
[100] M. Uehar, Y. Arai, S. Watanabe, H. Adlercreutz, Biofactors 12 (2000) 217.
[101] L.V. Glitso, W.M. Mazur, H. Adlercreutz, K. Wahala, T. Makela, B. Sandstrom, K.E. Bach Knudsen, Br. J. Nutr. 84 (2000) 429.
[102] E. Bowey, H. Adlercreutz, I. Rowland, Food Chem. Toxicol. 41 (2003) 631.
[103] K.E. Bach Knudsen, A. Serena, A.K. Kjaer, I. Tetens, S.M. Heinonen, T. Nurmi, H. Adlercreutz, J. Nutr. 133 (2003) 1368.

[104] M. Morton, O. Arisaka, A. Miyake, B. Evans, Environ. Toxicol. Pharmacol. 7 (1999) 221.
[105] J.W. Lampe, D.R. Gustafson, A.M. Hutchins, M.C. Martini, S. Li, K. Wahala, G.A. Grandits, J.D. Potter, J.L. Slavin, Cancer Epidemiol. Biomarkers Prev. 8 (1999) 699.
[106] M.S. Morton, O. Arisaka, N. Miyake, L.D. Morgan, B.A. Evans, J. Nutr. 132 (2002) 3168.
[107] P.B. Grace, J.I. Taylor, N.P. Botting, T. Fryatt, M.F. Oldfield, S.A. Bingham, Anal. Biochem. 315 (2003) 114.
[108] H. Kuo, W. Ding, J. Chromatogr. A 1027 (2004) 67.
[109] K.D. Setchell, M.S. Faughnan, T. Avades, L. Zimmer-Nechemias, N.M. Brown, B.E. Wolfe, W.T. Brashear, P. Desai, M.F. Oldfield, N.P. Botting, A. Cassidy, Am. J. Clin. Nutr. 77 (2003) 411.
[110] K.D. Setchell, N.M. Brown, P. Desai, L. Zimmer-Nechemias, B.E. Wolfe, W.T. Brashear, A.S. Kirschner, A. Cassidy, J.E. Heubi, J. Nutr. 131 (2001) 1362S.
[111] Q. Wu, M. Wang, J.E. Simon, J. Agric. Food Chem. 52 (2004) 2763.
[112] J. Maubach, M.E. Bracke, A. Heyerick, H.T. Depypere, R.F. Serreyn, M.M. Mareel, D. De Keukeleire, J. Chromatogr. B 784 (2003) 137.
[113] S.O. Duke, A.M. Rimando, P.F. Pace, K.N. Reddy, R.J. Smeda, J. Agric. Food Chem. 51 (2003) 340.
[114] T.J. Vyn, X. Yin, T.W. Bruulsema, C.J. Jackson, I. Rajcan, S.M. Brouder, J. Agric. Food Chem. 50 (2002) 3501.
[115] L. Krenn, I. Unterrieder, R. Ruprechter, J. Chromatogr. B 777 (2002) 123.
[116] B. Eisen, Y. Ungar, E. Shimoni, J. Agric. Food Chem. 51 (2003) 2212.
[117] B.F. Thomas, S.H. Zeisel, M.G. Busby, J.M. Hill, R.A. Mitchell, N.M. Scheffler, S.S. Brown, L.T. Bloeden, K.J. Dix, A.R. Jeffcoat, J. Chromatogr. B 760 (2001) 191.
[118] R. Mateos, J.L. Espartero, M. Trujillo, J.J. Rios, M. Leon-Camacho, F. Alcudia, A. Cert, J. Agric. Food Chem. 49 (2001) 2185.
[119] A.D. Muir, N.D. Westcott, J. Agric. Food Chem. 48 (2000) 4048.
[120] S.L. Lee, J.K. Ahn, S.H. Kim, J.T. Kim, S.J. Han, M.Y. Jung, I.M. Chung, J. Agric. Food Chem. 51 (2003) 3382.
[121] K.D. Setchell, S.J. Cole, J. Agric. Food Chem. 51 (2003) 4146.
[122] Y. Ungar, O.F. Osundahunsi, E. Shimoni, J. Agric. Food Chem. 51 (2003) 4394.
[123] M.I. Genovese, F.M. Lajolo, J. Agric. Food Chem. 50 (2002) 5987.
[124] T. Song, K. Barua, G. Buseman, P.A. Murphy, Am. J. Clin. Nutr. 68 (1998) 1474S.
[125] L.S. Hutabarat, H. Greenfield, M. Mulholland, J. Food Compos. Anal. 14 (2001) 43.
[126] L.S. Hutabarat, H. Greenfield, M. Mulholland, J. Chromatogr. A 886 (2000) 55.
[127] D.A. Roston, P.T. Kissinger, Anal. Chem. 55 (1981) 1695.
[128] Y. Kitada, Y. Ueda, M. Yamamoto, M. Ishikawa, H. Nakazawa, M. Fujita, J. Chromatogr. 366 (1986) 403.
[129] S.M. Lunte, J. Chromatogr. 2 (1987) 371.
[130] K.D.R. Setchell, M.B. Welsh, C.K. Lim, J. Chromatogr. 386 (1987) 315.
[131] D.J. Jones, C.K. Lim, D.R. Ferry, A. Gescher, Biomed. Chromatogr. 12 (1998) 232.
[132] W.R. Matson, P. Langlais, L. Volicer, P.H. Gamache, E. Bird, K.A. Mark, Clin. Chem. 30 (1984) 1477.
[133] P.H. Gamache, E. Ryan, I.N. Acworth, J. Chromatogr. 635 (1993) 143.
[134] P.H. Gamache, T.J. Maher, K.D.R. Setchell, T.H. Wu, I.N. Acworth, Am. J. Clin. Nutr. 68 (1998) 1536S.
[135] P.H. Gamache, I.N. Acworth, Proc. Soc. Exp. Biol. Med. 217 (3) (1998) 274.
[136] T. Nurmi, H. Adlercreutz, Anal. Biochem. 274 (1999) 110.
[137] T. Nurmi, W. Mazur, S. Heinonen, J. Kokkonen, H. Adlercreutz, J. Pharm. Biomed. Anal. 28 (2002) 1.
[138] T. Nurmi, S. Voutilainen, K. Nyyssonen, H. Adlercreutz, J.T. Salonen, J. Chromatogr. B 798 (2003) 101.
[139] T. Kraushofer, G. Sontag, J. Chromatogr. B 777 (2002) 61.
[140] L. Zubik, M. Meydani, Am. J. Clin. Nutr. 77 (2003) 1459.
[141] C. Gooijer, S.J. Kok, F. Ariese, Analusis 28 (2000) 679.
[142] H. Lingeman, A.J.G. Mank, C. Gooijer, Methodol. Surveys Bioanal. Drugs 24 (1996) 275.
[143] M. Richelle, S. Pridmore-Merten, S. Bodenstab, M. Enslen, E.A. Offord, J. Nutr. 132 (2002) 2587.
[144] W.M. Niessen, J. Chromatogr. A 1000 (2003) 413.
[145] E. Gelpi, J. Chromatogr. A 1000 (2003) 567.
[146] E. Gelpi, Int. J. Mass Spectrom. Ion Processes 118–119 (1992) 683.
[147] P. Arpino, Mass Spectrom. Rev. 11 (1992) 3.
[148] J. Sherma, J. AOAC Int. 86 (2003) 873.
[149] C.K. Lim, G. Lord, Biol. Pharm. Bull. 25 (2002) 547.
[150] L.F. Marvin, M.A. Roberts, L.B. Fay, Clin. Chim. Acta 337 (2003) 11.
[151] T. Bonk, A. Humeny, Neuroscientist 7 (2001) 6.
[152] J. Wang, P. Sporns, J. Agric. Food Chem. 48 (2000) 2807.
[153] J. Wang, P. Sporns, J. Agric. Food Chem. 47 (1999) 1549.
[154] G.A. Grant, S.L. Frison, J. Yeung, T. Vasanthan, P. Sporns, J. Agric. Food Chem. 51 (2003) 6137.
[155] S. Frison-Norrie, P. Sporns, J. Agric. Food Chem. 50 (2002) 2782.
[156] E. Gelpi, J. Mass Spectrom. 37 (2002) 241.
[157] W.M. Niessen, J. Chromatogr. A 856 (1999) 179.
[158] H. Hayen, U. Karst, J. Chromatogr. A 1000 (2003) 549.
[159] A. Lagana, A. Bacaloni, I. De Leva, A. Faberi, G. Fago, A. Marino, Anal. Chim. Acta 501 (2004) 79.
[160] K.D. Setchell, N.M. Brown, P.B. Desai, L. Zimmer-Nechimias, B. Wolfe, A.S. Jakate, V. Creutzinger, J.E. Heubi, J. Nutr. 133 (2003) 1027.
[161] N.C. Twaddle, M.I. Churchwell, D.R. Doerge, J. Chromatogr. B 777 (2002) 139.
[162] A.A. Franke, L.J. Custer, L.R. Wilkens, L.L. Le Marchand, A.M. Nomura, M.T. Goodman, L.N. Kolonel, J. Chromatogr. B 777 (2002) 45.
[163] D.B. Clarke, A.S. Lloyd, N.P. Botting, M.F. Oldfield, P.W. Needs, H. Wiseman, Anal. Biochem. 309 (2002) 158.
[164] N.M. Saarinen, A. Smeds, S.I. Makela, J. Ammala, K. Hakala, J.M. Pihlava, E.L. Ryhanen, R. Sjoholm, R. Santti, J. Chromatogr. B 777 (2002) 311.
[165] A. Smeds, K. Hakala, J. Chromatogr. B 793 (2003) 297.
[166] P.B. Grace, J.I. Taylor, N.P. Botting, T. Fryatt, M.F. Oldfield, N. Al-Maharik, S.A. Bingham, Rapid Commun. Mass Spectrom. 17 (2003) 1350.
[167] D.A. van Elswijk, U.P. Schobel, E.P. Lansky, H. Irth, J. van der Greef, Phytochemistry 65 (2004) 233.
[168] M.A. Rostagno, M. Palma, C.G. Barroso, J. Chromatogr. A 1012 (2003) 119.
[169] E. Federici, A. Touche, S. Choquart, O. Avanti, L. Fay, E. Offord, D. Courtois, Phytochemistry 64 (2003) 717.
[170] F. Rafii, C. Davis, M. Park, T.M. Heinze, R.D. Beger, Arch. Microbiol. 180 (2003) 11.
[171] N.G. Coldham, C. Darby, M. Hows, L.J. King, A.Q. Zhang, M.J. Sauer, Xenobiotica 32 (2002) 45.
[172] H. Wiseman, K. Casey, D.B. Clarke, K.A. Barnes, E. Bowey, J. Agric. Food Chem. 50 (2002) 1404.
[173] N. Fang, S. Yu, T.M. Badger, J. Agric. Food Chem. 50 (2002) 2700.
[174] W.H. Tolleson, D.R. Doerge, M.I. Churchwell, M.M. Marques, D.W. Roberts, J. Agric. Food Chem. 50 (2002) 4783.
[175] A.N. Begum, C. Nicolle, I. Mila, C. Lapierre, K. Nagano, K. Fukushima, S.M. Heinonen, H. Adlercreutz, C. Remesy, A. Scalbert, J. Nutr. 134 (2004) 120.
[176] J.P. Antignac, R. Cariou, B. Le Bizec, J.P. Cravedi, F. Andre, Rapid Commun. Mass Spectrom. 17 (2003) 1256.

[177] L. Valentin-Blasini, B.C. Blount, H.S. Rogers, L.L. Needham, J. Expo. Anal. Environ. Epidemiol. 10 (2000) 799.
[178] L. Valentin-Blasini, B.C. Blount, S.P. Caudill, L.L. Needham, J. Expo. Anal. Environ. Epidemiol. 13 (2003) 276.
[179] C.J. Liu, J.W. Blount, C.L. Steele, R.A. Dixon, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 14578.
[180] J.K. Prasain, R. Patel, M. Kirk, L. Wilson, N. Botting, V.M. Darley-Usmar, S. Barnes, J. Mass Spectrom. 38 (2003) 764.
[181] J.K. Prasain, K. Jones, M. Kirk, L. Wilson, M. Smith-Johnson, C. Weaver, S. Barnes, J. Agric. Food Chem. 51 (2003) 4213.
[182] E. Bayer, K. Albert, M. Nieder, E. Grom, T. Keller, J. Chromatogr. 186 (1979) 479.
[183] J.F. Haw, T.E. Glass, D.W. Hausler, E. Motell, H.C. Dorn, Anal. Chem. 52 (1980) 1135.
[184] K. Albert, M. Nieder, E. Bayer, M. Spraul, J. Chromatogr. 346 (1985) 17.
[185] C. Dalvit, G. Shapiro, J.M. Bohlen, T. Parella, Magn. Res. Chem. 37 (1999) 7.
[186] S.C. Bobzin, S. Yang, T.P. Kasten, J. Chromatogr. B 748 (2000) 259.
[187] S.X. Peng, Biomed. Chromatogr. 14 (2000) 430.
[188] O. Corcoran, M. Spraul, Drug Discov. Today 8 (2003) 624.
[189] J.L. Wolfender, K. Ndjoko, K. Hostettmann, J. Chromatogr. A 1000 (2003) 437.
[190] J.L. Wolfender, K. Ndjoko, K. Hostettmann, Phytochem. Anal. 12 (2001) 2.
[191] J. Fritsche, R. Angoelal, M. Dachtler, J. Chromatogr. A. 972 (2002) 195.
[192] E.F. Queiroz, J.L. Wolfender, K.K. Atindehou, D. Traore, K. Hostettmann, J. Chromatogr. A 974 (2002) 123.
[193] Y. Zhao, A. Nookandeh, B. Schneider, X. Sun, J. Stoeckight, J. Chromatogr. A 837 (1999) 83.
[194] A. Cavin, O. Potterat, J.L. Wolfender, K. Hostettmann, J. Nat. Prod. 61 (1998) 1497.
[195] R.A. Frazier, A. Papadopoulou, Electrophoresis 24 (2003) 4095.
[196] X. Gu, Q. Chu, M. O'Dwyer, M. Zeece, J. Chromatogr. A 881 (2000) 471.
[197] J.W. Jorgenson, K.D. Lukcas, Anal. Chem. 53 (1981) 1298.
[198] J.W. Jorgenson, K.D. Lukcas, J. Chromatogr. 218 (1981) 209.
[199] T. Aussenac, S. Lacombe, J. Dayde, Am. J. Clin. Nutr. 68 (1998) 1480S.
[200] S.P. Wang, K.J. Huang, J. Chromatogr. A 1032 (2004) 273.
[201] M. Vaher, M. Koel, J. Chromatogr. A 990 (2003) 225.
[202] P.C. Dabas, P. Bancora, C.M. Vescina, J. Capill. Electrophor. 8 (2003) 53.
[203] L.Y. Gao, Q.C. Chu, J.N. Ye, Food Chem. 78 (2002) 255.
[204] Q.C. Chu, W.Q. Qu, Y.Y. Peng, Q.H. Cao, N.J. Ye, Chromatographia 58 (2003) 67.
[205] B.R. Baggett, J.D. Cooper, E.T. Hogan, J. Carper, N.L. Paiva, J.T. Smith, Electrophoresis 23 (2002) 1642.
[206] T.F. Jiang, X. Du, Y.P. Shi, Chromatographia 59 (2004) 255.
[207] V. Brandolini, A. Maietti, P. Tedeschi, E. Durini, S. Vertuani, S. Manfredini, J. Agric. Food Chem. 50 (2002) 7407.
[208] J.A. Starkey, Y. Mechref, C.K. Byun, R. Steinmetz, J.S. Fuqua, O.H. Pescovitz, M.V. Novotny, Anal. Chem. 74 (2002) 5998.
[209] O. Lapcik, J. Stursa, T. Kleinova, M. Vitkova, H. Dvorakova, B. Klejdus, J. Moravcova, Steroids 68 (2003) 1147.
[210] C. Bennetau-Pelissero, C. Le Houerou, V. Lamothe, F. Le Menn, P. Babin, B. Bennetau, J. Agric. Food Chem. 48 (2000) 305.
[211] C. Le Houerou, C. Bennetau-Pelissero, V. Lamothe, F. Le Menn, P. Babin, B. Bennetau, Tetrahedron 56 (2000) 295.
[212] C. Bennetau-Pelissero, B. Arnal-Schnebelen, V. Lamothe, P. Sauvant, J.L. Sagne, M.A. Verbruggen, J. Mathey, O. Lavialle, Food Chem. 82 (2003) 645.
[213] R. L'homme, E. Brouwers, N. Al-Maharik, O. Lapcik, R. Hampl, H. Mikola, K. Wahala, H. Adlercreutz, J. Steroid Biochem. Mol. Biol. 81 (2002) 353.
[214] E. Brouwers, R. L'homme, N. Al-Maharik, O. Lapcik, R. Hampl, K. Wahala, H. Mikola, H. Adlercreutz, J. Steroid Biochem. Mol. Biol. 84 (2003) 577.