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Review

Identification and quantification of polyphenol phytoestrogens in foods and human biological fluids

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Abstract

We review the methods used to measure phytoestrogens (genistein, daidzein, lignans and their derivatives) in foods and biological fluids, and discuss advantages and disadvantages of each. The range of detection limits reported varies widely between individual laboratories, but generally the best reported sensitivity is as follows: immunoassay>HPLC–mass spectrometry=HPLC–multichannel electrochemical detection (coularray)>GC–single ion monitoring-mass spectrometry> HPLC–UV diode array>HPLC–single channel electrochemical detection. The best sensitivity reported so far is 0.002 pmol per assay for daidzein by radioimmunoassay. HPLC with UV diode array detection is the most commonly employed, but is the least sensitive and specific. GC and HPLC coupled with mass spectrometry or electrochemical detection are the most accurate and reproducible methods for a wide variety of analytes. Generally most methods, with the exception of immunoassay, have not been correlated with other methods. Recoveries from extraction methods, limits of detection, nature of compounds analysed and the internal standards used are summarised for more than 90 reports in the literature. From this data, it is clear that an inter-laboratory validation and correlation between a wide range of methods for phytoestrogen analysis is required. One underdeveloped area that requires particular attention is the analysis of plant lignans.

Keywords: Reviews; Polyphenol phytoestrogens

Contents

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1. Introduction

Polyphenol phytoestrogens are biologically active non-nutrient dietary compounds that may have a beneficial influence on human health [1,2] though concerns have also been expressed of potential deleterious effects [3]. To help understand the full consequences of the biological activity of polyphenol phytoestrogens, various analytical techniques have been utilised for their measurement in food matrices and human biological fluids.

Analysis of biological fluids for polyphenol phytoestrogens increases the knowledge of their metabolism and bioavailability both of which are essential for elucidating their biological activity. In addition accurate determination of levels in foods helps to correlate epidemiological and intervention study data with health outcome.

The main recognised polyphenol phytoestrogens are the isoflavonoids and lignans and this article will assess the main reported analytical methodologies. Aspects of method performance such as limits of detection and inter-/intra-assay variation, as well as a brief discussion of the benefits and limitations of particular methods, are presented. In addition, comments will be made about sample preparation and about the most appropriate analytical method to an analytical situation.

2. Main analytical methods

A broad range of analytical techniques has been applied to the analysis of polyphenol phytoestrogens in foods and human biological fluids during the last decade (Table 1). For food analysis, the methods that have been applied to the analysis of soy foods, which primarily contain genistein and daidzein derivatives, are described in Table 2. Other foods, containing a wider variety of polyphenol phytoestrogens are described in Table 3 and methods used for the analysis of human biological fluids are described in Tables 4 and 5.

Table 1
Most common analytical techniques for the analysis of polyphenol phytoestrogens in foods and human biological fluids

Technique	Type
Gas chromatography–mass spectrometry (GC–MS)	Isotope dilution selected ion monitoring (ID-GC–MS-SIM) Selected ion monitoring (GC–MS-SIM)
Liquid chromatography	UV detection (HPLC–UV) Diode array detection (HPLC–DAD) Electrochemical detection (HPLC–ED) Single cell Multiple coulometric (HPLC–coularray)
Liquid chromatography–mass spectrometry (LC–MS)	Electrospray ionisation (ESI), Atmospheric pressure chemical ionisation (APCI)
Immunoassay	Radioimmunoassay (RIA) Time resolved fluorescence immunoassay (TR-FIA) Enzyme-linked immunosorbent assay (ELISA)

Table 2
Analysis of soy, soy foods and soy supplements

Method	Compounds	Internal standards	Recovery (%)	Limit of detection (μg/kg)	Ref.
GC–MS-SIM	D, G	Dz, Gz	D=83–85 G=91–95	100 (limit of quantification)	[4]
HPLC–UV C_8 25×0.5 cm Gradient	D, G, Gl, Dz, Gz, Gn MDz, MGz, MGn ADz, AGz, AGn	Equilinen [5,7] fluorescein [6]	90–93 [6]	Not quoted	[5–7]
HPLC–UV C_{18} 25×0.5 cm Gradient	D, G, Gl, Dz, Gz, Gn MDz, MGz, MGn ADz, AGz, AGn	Isoflavone F	94	Solid food: 1000 Liquid soy foods: 1000 (μg/l)	[8]
HPLC–UV C_{18} 25×0.5 cm Isocratic	D, G		D=94 G=95	100	[9]
HPLC–UV C_{18} 25×0.5 cm Gradient	D, G, Gl, Dz, Gz, Gn MDz, MGz, MGn ADz, AGz, AGn	Apigenin	Apigenin=98.5 Isoflavone standards=99–101	500	[10]
HPLC–DAD C_{18} 15×0.5 cm Gradient	D, G, Gl, Dz, Gz, Gn MDz, MGz MGn, ADz, AGz, AGn	Flavone		200–500	[11]
HPLC–DAD C_{18} 25×0.5 cm Gradient	D, G, Gl, Dz, Gz, Gn MDz, MGz MGn, ADz, AGz, AGn	THB (12–15) External standards; D, G, Gz (13–15)	THB 94–98 D, G, Gz 81–98	Not quoted except in [14] D=900 G=500	[12–15]
HPLC–DAD C_{18} 25×0.5 cm Gradient	D, G, Gl, Dz, Gz, Gn	Flavone	60–119 of samples spiked with D, G, Gl, Dz, Gz Gn	Solid food: 500–3300 Soy milk: 109–660 (μg/l) Soy sauce: 11–66 (μg/l)	[16]
HPLC–DAD C_{18} 25×0.5 cm Gradient	D, G, Dz, Gz, MDz, MGz, ADz, AGz,	ISTD	>90 (18)	100 μg/l	[17(a),18]

Table 2. Continued

Method	Compounds	Internal standards	Recovery (%)	Limit of detection (μg/kg)	Ref.
HPLC–DAD C_{18} 25×0.5 cm Gradient	G, Gz		G=90, Gz=91	100	[19]
HPLC–DAD Phenyl 15×0.4 cm Isocratic	D, G, B, F, C		89–104	600–3000	[20,21]
HPLC–ED C_{18} 25×0.5 cm Isocratic	D, G, B	Bisphenol A	72–94	900–1000	[22]
Multiple coulometric	D, G		69–86	900–1400	[23]
HPLC–ESI-MS C_{18} 25×0.5 cm Gradient	D, G	Dz, Gz	D=76–84 G=89–92	D=200 G=700	[24]

Notes: D=daidzein, Dz=daidzin, ADz=acetyl-daidzin, MDz=malonyl-daidzin, G=genistein, Gz=genistin, AGz=acetyl-genistin, MGz=malonyl-genistin, Gl=glycitein, Gn=glycitin, AGn=acetyl-glycitin, MGn=malonyl-glycitin, B=biochanin A, F=formononetin, C=coumestrol, THB=2,4,4′-trihydroxydeoxybenzoin, ISTD=3-isobutyl-1-methylxanthin. (a) Capillary zone electrophoresis method also reported.

Table 3
Analysis of non-soy foods

Method	Compounds[a]	Internal standards	Recovery (%)	Limit of detection (μg/kg)	Ref.
ID-GC–MS-SIM	D, G, B, F, C, M, SECO[b]	Deuterated isoflavone and lignan	96–105	20–30	[30–33]
GC–MS-SIM	D, G	Dz, Gz	70–109	Limit of quantification	
			0.1	0.2 [34] 1.0 [35]	[34,35]
	M, SECO, Sh	Anthraflavic acid added at derivatisation		SECO+Sh 1000–100 000 M 10–10 000 Quantitative range	[37]
HPLC–ED	D, G, E	EGU	70	D, G, 2 (μg/l) E, 4 (μg/l)	[38]
HPLC–DAD	D, G, B, F, C	Flavone	94–105	13–70	[27][c]
HPLC–HN-APCI-MS	D, G, B, F, C, SECO, M	4MU[a]		250	[39][c]

[a] D=Daidzein, Dz=daidzin, G=genistein, Gz=genistin, B=biochanin A, F=formononetin, C=coumestrol, M=matairesinol, SECO=secoisolariciresinol, Sh=shonanin chemically identical to anhydrosecoisolariciresinol [37], EGU=17-β-estradiol glucuronide, 4MU=4-methylumbelliferyl.

[b] Acid hydrolysis causes SECO to convert to shonanin which also occurs naturally [37]. The value for SECO is the sum of both compounds.

[c] Soy foods also analysed.

2.1. Soy foods

Soy is the most common food to have been analysed for polyphenol phytoestrogens because this is the richest dietary source of daidzein and genistein whose biological activities have been extensively studied [1,2]. Table 2 describes analytical techniques reported in the literature, the compounds analysed, the internal standards used, their percentage recovery and limits of detection of the analytes in soy foods. The main analytical techniques are high-performance liquid chromatography with UV or diode array detection (HPLC–DAD) using reversed-phase C_{18} stationary phases with gradient elution (Tables 2 and references therein). Other less common techniques, not included in the tables, have been described for the measurement of isoflavonoids in soy such as capillary zone electrophoresis (CZE) [17], matrix-

Table 4
Analysis of human body fluids: urine

Method	Compounds	Internal standards	Recovery (%)	Limit of detection (nmol/l)	Ref.
ID-GC–MS-SIM	D, G, E, M, DMA Ent 1, Ent 2	Deuterated isoflavone and lignan	97–106	3 (a)	[54,55]
GC–MS-SIM	D, G, B, F	Free=6-HF Total=kaempferol	70–90 except B 57–64	4–10	[56]
HPLC–DAD C_{18} 15×0.5 cm Gradient	D, G, E, C, DMA D, G	Flavone		50–164 D=63 G=36	[57] (b, c) [58] (b)
HPLC–DAD C_{18} 30×0.4 cm Gradient	D, G, Gl	THB	D= 73–80 G= 83–89 Gl= 57–66	50	[59] (d)
HPLC–ED C_{18} 30×0.4 cm Multiple coulometric	D, Dz, G, E, C, Ent 1, Ent 2		85–96	1.6–7	[60]
HPLC–HN-APCI-MS C_{16} 25×0.5 cm Isocratic	D, DHD, G, DHG DMA, Sulfate and glucuronide conjugates (f)	B	88–93	20 except E 800	[62]
HPLC–HN-APCI-MS–MS Keystone Prism 50×3 mm Isocratic	D, G, M, C, E, DMA Ent 1, Ent 2	Deuterated isoflavone and lignan, 4-MU glucuronide, 4-MU sulfate (external standards: 4-nitrophenol, hexestrol)	92–104	0.5–40	[63] (e)

Table 4. Continued

Method	Compounds	Internal standards	Recovery (%)	Limit of detection (nmol/l)	Ref.
Immunogen	Target analyte (Cross reactants>5%)				
RIA Daidzein-4′-*O*-carboxymethyl ether-BSA	D (F, 60%)		87–105	0.02 (0.3–40)*	[64] (g)
TR–FIA Daidzein-4′-*O*-carboxymethyl ether-BSA	D (F, 206%) (Dz, 6%)		94	0.4 (1–216)	[65] (g)
TR–FIA Daidzein-7′-*O*-carboxymethyl ether-KLH	D		80–90	2	[66]
TR-FIA Genistein-4′-*O*-carboxymethyl ether-BSA	G (B, 500%) (F, 44%) (DHG, 11%) (Gz, 8%)		89	0.4 (2–370)	[65] (g)
ELISA 6-carboxymethyl genistein-KLH	G (B, 220%)		69–121	185 (40–1200)	[67] (d)

E=Equol, DMA=*O*-desmethylangolensin, Ent 1=enterolactone, Ent 2=enterodiol, 6-HF=6-hydroxyflavon, DHD=dihydrodaidzein, DHG=dihydrogenistein, RIA=radioimmunoassay, TR-FIA=time-resolved fluorescent immunoassay, ELISA=enzyme-linked immunosorbent assay, BSA=bovine serum albumin, KLH=keyhole limpet haemocyanin.

(a) Assuming 1.5 l excreted in 24 h.

(b) Plasma and human milk also analysed [61].

(c) A single coulometric electrochemical detector was used in tandem with DAD in this method for confirmation purposes and lower detection limits (16–85 nmol/l).

(d) Plasma also analysed.

(e) Method also applied to serum with recoveries of 81–101% and a limit of detection of 0.3–10 nmol/l.

(f) Following selective conjugate hydrolysis with β-glucuronidase or sufatase.

(g) Method also used for serum/plasma analyses. In these assays [^{3}H]estradiol-17β-glucuronide was used as an internal standard.

*Denotes working range (nmol/l).

assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) [25] and a microtitration plate based assay exploiting the human estrogen receptor α (ER α) [26]. The CZE method described by Mellenthin and Galensa was compared with their HPLC–DAD method, which was found to be more sensitive [17,18]. The human estrogen receptor based assay [26] was also validated against a HPLC–DAD method. A good correlation (r^2=0.9) was found between the two techniques for the analysis of genistein. This receptor-based microtitration plate assay can also be used to screen foods for potential phytoestrogen content.

2.1.1. Sample preparation and extraction

In soy daidzein, genistein and a third less abundant isoflavone glycitein may exist in four forms; aglycones, β-glucosides, acetyl-β-glucosides and

malonyl-β-glucosides. The total levels of these isomers have been reported to range from 0.5 to 4 g/kg [2]. Malonyl-β-glucosides predominate in soybeans but food processing causes changes to the relative proportions of each form because of chemical lability of the malonyl and acetyl derivatives [13,15].

Hydrolytic [4,9,16,20–24] and non-hydrolytic methods for isoflavone extraction have been used [5–8,10–19]. Hydrolytic methods are needed when GC–MS single ion monitoring (SIM) is used for analysis because it is easier to form volatile silanyl derivatives from the aglycones than the glycosides. To form the aglycones, hydrolysis with 1–2 *M* hydrochloric acid at 100 °C or refluxing with acid in the presence of ethanol has been used. However there are some reports indicating genistein to be unstable under acid hydrolysis conditions [26,27]. Milder alternatives are enzymatic hydrolysis by β-glucuronidase/arylsulfatase from *Helix pomatia*; β-glycosidase from almonds, or a crude extract of cellulase from *Aspergillus niger* [4,27]. The use of this cellulase preparation has been suggested to be the most suitable hydrolytic method and more reproducible when compared to acidic hydrolysis [4].

Table 5
Analysis of human body fluids: blood, serum, plasma

Method	Compounds	Internal standards	Recovery (%)	Limit of detection (nmol/l)	Ref.
ID-GC–MS-SIM (a)	D, G, E, M, DMA Ent 1, Ent 2	Deuterated isoflavone and lignan	86–99	0.2–1.0 1–3	[68,69] [70]
ID-GC–MS-SIM (b)	D, G, E, Ent 1, Ent 2	Deuterated isoflavone and lignan		0.04–0.2 [71]	[71,72]
ID-GC–MS-SIM (c)	D, G, Gl, B, E, F	[^{13}C]D, [^{13}C]G, DHF		Not quoted	[7]
GC–MS-SIM	D, G, E, DMA, DHD	DHF		Not quoted	[5]
HPLC–ED C_{18} 25×0.4 cm Single cell (750 mV)	D, G, E	EGU	72–78	200	[77,78] (d)
HPLC–ED C_{18} 15×0.3 cm Gradient Multiple coulometric	D, Dz, DHD, E, DMA, G, Gz, DHG SECO, M, Ent 1, Ent 2 Sh	EGU	68–91	90–900	[79]
HPLC–HN-APCI-MS C_8 10×0.5 cm Isocratic	D, DHD, G, DMA	Phenol-phthalein-glucuronide, 4-MUS, B	90	1	[80]
HPLC–ES-MS Luna C_{18} 150×2 mm Isocratic	D, G, sulfate and glucuronide conjugates (e)	Deuterated D and G	D=44–95 G=66–106	5 15 (limit of quantitation)	[81,82]

Table 5. Continued

Method	Compounds	Internal standards	Recovery (%)	Limit of detection (nmol/l)	Ref.
Immunogen	Target analyte (Cross reactants>5%)				
RIA Genistein-4′-*O*-carboxymethyl ether-BSA	G (B, 173%) (D, 6%)			0.02 (0.3–7)*	[83]
RIA Genistein-7′-*O*-carboxymethyl ether-BSA	G (Gz, 26%) (D, 6%)			0.05 (0.3–7)	[83]
TR–FIA 5′-*O*-carboxy-methoxy-enterolactone-BSA	Ent 1	[^{3}H]EGU		Method 1, 0.3 (1.5–540) Method 2 (short), 1.5	[84–86] (a)
ELISA Daidzein-2-carboxylic acid KLH	D		>90	0.8 (2–4000)	[87,88]
ELISA Equol-7-*O*-carboxybutyl-KLH	E (E-4′-ME, 200%) (E-7,4′-DME, 110%)			3 (20–1400)	

DHF=Dihydroxyflavone, E-4′-ME=equol-4′-methyl ether, E-7,4′-DME=equol-7,4′-dimethylether, 4-MUS=4-methylumbelliferyl sulfate.

(a) This method has also been used to analyse cord blood and amniotic fluid.

(b) This method has also been used to analyse soy foods, urine, prostatic fluid, human milk, nipple aspirate fluid and breast tissue, but no recoveries, limits of detection or inter-/intra-assay variability was reported for these differing matrices [73–76].

(c) Method also applied to urine.

(d) Urine also analysed.

(e) Following selective conjugate hydrolysis with β-glucuronidase or sufatase.

*Denotes working range (nmol/l).

For analysis of soy foods for isoflavonoids, the recent trend has been to avoid hydrolysis in order to obtain information about the quantity and type of isoflavone derivative consumed, as these may have differing pharmacokinetics and bioactivity [28], to simplify the extraction and overall analytical procedure and avoid any possible degradation of aglycones. Typical extraction conditions that have been used are stirring–freeze–dried powdered samples with methanol–water (80:20, v/v) at room temperature or 4 °C, or with a mixture of acetonitrile–hydrochloric acid (0.1 *M*)–water [10,12–15]. Using acidified solvents has been recommended by the compilers of the US Department of Agriculture—Iowa State University Database [29]. An alternative solvent of acetonitrile–water and dimethyl sulfoxide (DMSO) has been suggested recently [10]. In this report, a comparison was made with extraction by

acidified acetonitrile or aqueous methanol. Although slightly greater amounts of the different isoflavone derivatives were extracted with the newer solvent system, compared with acidified acetonitrile, greater overall reproducibility was attained with the latter. It should be noted that when using acidified acetonitrile to extract different soy foods the quantity of water used for extraction may also have to be varied [15].

2.1.2. Recovery of added internal standards

Most methods listed in Table 2 report the use of internal standards to evaluate analyte loss during the extraction process. The tabulated recoveries are generally greater than 90%. The standards must have similar chemical properties to isoflavones and elute in discrete regions of the HPLC chromatogram which, providing that the chosen compound has a stable highly reproducible retention time, enables their use as reference peaks for the correct identification of analyte peaks and aid in analyte quantification. Examples of compounds that have been used are flavone, apigenin and trihydroxydeoxybenzoin (THB) [10–15]. Of these THB is not commercially available and has to be synthesised. Addition of isoflavones as standards is now more frequently reported [4,8,24]. Some reports indicate that recovery of added standards can vary with the types of soy food matrix analysed [16]. Quality control measures for the routine analysis of soy isoflavones by HPLC–DAD, as described by Murphy et al. [15] for their method and promoted as a reference method, are now being undertaken in the description of new methods [10].

2.2. Non soy foods

These have recently been examined for their isoflavonoid and lignan content in order to obtain an understanding of possible dietary sources of isoflavonoids that are present in typical Western diets that do not normally include a high level of soy. Isoflavonoid levels in non-soy foods are much lower than in soy [30–33]. To measure these low levels, sensitive analytical methods are required. Table 3 shows the analytical methods that have been applied to non-soy foods. The HPLC methods that use some form of UV detection have been considered not sufficiently sensitive for the analysis of the expected low levels of isoflavonoids [30] but the method reported by Franke et al. [27] (30–70 μg/kg) appears to be as sensitive as the isotope dilution (ID) GC–MS-SIM technique [30–33] (20–30 μg/kg). The lowest limit of quantification (0.1 μg/kg) was reported for a GC–MS-SIM method [34] but this was only applied to the analysis of daidzein and genistein. Foods other than soy such as chickpeas (garbanzo beans, *Cicer arietinum*) are known to contain biochanin A and formononetin and these may be the predominant isoflavonoids. In this respect, as more isoflavones are analysed, the ID-GC–MS-SIM method [30–33] can provide more information.

Another technique [36], not included in Table 3, is the combined use of HPLC with radio-immunoassay to analyse beer for daidzein, genistein, biochanin A and formononetin. Here, because of the cross-reactivity of the antisera (for further discussion see Section 2.4), isoflavonoids in beer were first separated by HPLC and collected fractions analysed individually. Limits of detection were 0.08 nmol/l for daidzein and formononetin and 0.15 nmol/l for genistein and biochanin A. Inter- and intra-assay relative standard deviations ranged from 10 to 13% and 6–7%, respectively.

2.2.1. Sample extraction and preparation

Of the methods listed in Table 3, none have been used to determine isoflavonoid glycosides. This is because the exact nature and composition of isoflavone glycosides present in foods other than soy is unknown and so all the reported sample preparation protocols utilise a hydrolysis step to form isoflavonoid aglycones. Such a step is also necessary to produce derivatisable analytes for GC–MS. Before analysis by ID-GC–MS-SIM, a time consuming and complex sample preparation consisting of several solid-phase extraction and chromatographic separations is used. The sample preparation protocol used before analysis in the second GC–MS method [34,35] is simpler but still involves four steps before reaction with the silanisation reagent. The simplest sample preparation protocol was described for the HPLC–DAD method [27] though this involves acidic hydrolysis and may suffer the limitations as mentioned above for soy food analysis.

2.2.2. Internal standards

These have been included in procedures recorded in Table 3 and recoveries are generally greater than 90%. For the ID-GC–MS-SIM method possible losses during sample clean up are evaluated by the addition of deuterated internal standards. The potential for exchange with hydrogen of some of the deuterated sites of these standards has been a criticism of this procedure [40] though more stable standards have been synthesized [41–45]. In addition to the more stable deuterated phytoestrogen standards, the synthesis of singly ^{13}C-labelled isotopes of genistein, daidzein, biochanin A and formononetin has been described [46]. The ^{13}C-labelled isotopes can also be used in metabolism studies. A draw back of the currently available isotopically labelled compounds is that they are not available as glycosides. This would benefit phytoestrogen analysis because such standards would enable a better assessment of analyte losses occurring in hydrolytic reactions utilised in sample preparation procedures for ID-GC–MS-SIM. This problem was overcome in the alternative GC–MS method [34,35] by the addition of the glycosides daidzin and genistin which are commercially available unlike the isotopically labelled standards that are not generally available from commercial sources and need to be synthesized.

A descriptor of assay performance, not quoted in the Tables 2 and 3 but which has been assessed for the majority of the tabulated methods, is the percentage of inter- and intra-assay variation. For soy food analysis these generally fall within the range 2–16% (inter-assay) and 1–7% (intra-assay) and for non-soy foods the respective ranges are 5–17% and 2–8%.

2.3. Analysis of lignans in foods

Lignans are compounds, derived from plants, possessing a 2,3-dibenzylbutane skeleton and are associated with dietary fibre. The main dietary lignans to have been studied are secoisolariciresinol (SECO) and matairesinol (MAT) which are transformed by human intestinal bacteria to the mammalian lignans enterodiol and enterolactone. On further oxidation enterodiol is converted to enterolactone. These compounds have been suggested to have potential health benefits [1]. This has led to an increased use of lignan rich plants such as flaxseed (linseed), sunflower seed, rye, oats and barley in foods such as bakery products to provide ‘‘health added’’ products [47].

Additional analytical techniques for lignans, not mentioned in Tables 2 and 3, are reviewed and discussed in a recent article by Meagher and Beecher [47]. This report highlights a difference between the higher levels of mammalian lignans measured following in vitro fermentation of food stuffs with human faecal microbial cultures or when human biological fluids are analysed, compared to the amounts of plant lignan precursors determined in foods by chemical extraction and physico–chemical or immunoassay analysis [47,48]. It is possible that the discrepancy arises from the mammalian lignans being formed from plant lignan precursors other than SECO or MAT. Alternatively there could be inefficient extraction of SECO or MAT from certain plant materials during the hydrolysis stage of the extraction processes. Degradation of SECO may also occur during acid hydrolysis [37]. The degree of degradation is influenced by food type and hydrolysis time. In the ID-GC–MS-SIM protocol of Mazur et al. [30], this degradation would not be taken into account as the deuterated lignan internal standards are added after the acid hydrolysis step of their sample preparation procedure. Whatever the reasons, further research is needed to address the problem of lignan analysis and their quantification in food plants.

2.4. Human biological fluids

Following ingestion, plant isoflavonoids and lignans are absorbed and metabolised. The full extent of the metabolites produced from the parent compounds is still being examined and new information reported. An understanding of the range of polyphenolic phytoestrogen metabolites, major and minor, is of great interest because some metabolites have been shown to have more potent biological activity than the parent compound, for instance equol compared with daidzein [2,49,50]. The main analytical technique being used to investigate the spectrum of isoflavonoid and lignan metabolites is GC–MS [51–53].

The principal human biological fluids that have been analysed are urine and blood (serum or plasma). Tables 4 and 5 show the main techniques to have been used in recent years to analyse urine and blood for isoflavonoids and the mammalian lignans, enterolactone and enterodiol.

GC–MS techniques that have been used to analyse human biological fluids are sensitive and limits of detection (when quoted) range between 0.04 to 10 nmol/l. The tabulated methods use different sample pre-analysis purification procedures (see below) and assay performance characteristics such as limits of detection, recoveries and inter-/intra-assay variation have not always been quoted in the literature. In this regard the most extensively characterised has been the ID-GC–MS-SIM method used in the laboratory of Adlercreutz and co-workers [54,55,68–70]. For the analysis of serum, a study of the reliability of the ID-GC–MS-SIM method of Adlercreutz over a 2-year period has been reported [89]. There was a high intra and ''total assay'' variability for all analytes except enterolactone and genistein but this high variation probably reflects the low levels of analytes in the study samples where geometric means were less than 6 nmol/l for all analytes except enterolactone (20 nmol/l). Reliability data for other GC–MS methods have been reported for assays performed over 2 or 1.5 years [5,7]. For the 2 year analysis [5] inter-assay variation ranged from 8 to 11% for a serum sample containing 280 nmol/l isoflavone and in the later study [7], the mean inter-assay variation was 6% for daidzein and genistein for samples containing an average of 570 nmol/l of daidzein or genistein. Inter-assay variation was 10% for equol (25 nmol/l).

Though methods using HPLC with UV detection have been commonly applied to the analysis of soy foods, they have been used less frequently for the analysis of human body fluids. Franke et al. [57] describe the use of HPLC–DAD for urine, plasma and human milk. An earlier report by these authors [90] describes mobile phase solvents of acetonitrile and acetic acid–water which was later changed to a solvent system where methanol and dichloromethane were added to acetonitrile [57,61]. Though this resulted in a better separation of the analytes and improved limits of detection for equol and *O*-desmethylangolensin (DMA), those for daidzein, genistein and coumestrol were reduced. To improve detection limits for the new elution conditions the DAD system was linked in series with a single cell coulometric electrochemical detector, which was also used to confirm the identity of the peaks detected by DAD. In the case of human milk analysis, confirmation was provided by GC–MS-SIM. A recent report, of urine analysis, also shows differing detection limits for genistein and daidzein (26.6 and 54.3 nmol/l [57] compared with 35.8 and 62.8 nmol/l [58]).

HPLC–DAD has also been applied to the analysis of urine and plasma by Zhang et al. [59] with similar detection limits to those reported by Franke et al. [57]. The tabulated recovery values are for urine [59]. Recoveries from plasma were similar for daidzein and glycitein but lower for genistein (72% compared to 86%).

Coularray (multiple coulometric electrochemical cells) is a promising detection system when combined with HPLC, for the analysis of isoflavonoids and lignans in biological fluids. Gamache and Ackworth [60] applied coularray detection to urine analysis and reported detection limits comparable to GC–MS analysis and recoveries ranged from 85 to 96% for urine spiked with analytes. Nurmi and Adlercreutz [79] have used this detection system to analyse 13 polyphenol phytoestrogens in plasma. Detection limits, for an injection volume of 10 μl, ranged from 9 to 90 fmol with a wide range of linearity however these detection limits (when expressed as nmol/l) were higher than those reported by Gamache and Ackworth [60] for urine analysis and the range of recoveries was wider. Intra-assay variation was less than 4% for phytoestrogens [60]. For plasma analysis intra-assay variation was generally 10–15%, with a range of 1.5%, for enterolactone, to 42% for enterodiol [79]. In this report inter-assay variation was cited as 10–44%. The coularray detection system is claimed to have the advantages of sensitivity, a wide analytical range and selectivity because compounds that co-elute in the chromatogram may be distinguished on the basis of differing oxidation/reduction profiles (voltammograms [22]). A potential disadvantage is that when using electrochemical detectors the purity of the chemicals used for the mobile phase utilized in conjunction with reverse phase silica HPLC columns

is very important particularly when the full sensitivity of the detection system is being applied to the determination of trace analyte levels as, for instance, would be encountered in the analysis of isoflavone levels in the plasma of individuals commonly consuming a normal western diet.

In addition to being used in conjunction with GC, mass spectrometry has been used with liquid chromatography to analyse human biological fluids for polyphenol phytoestrogens. Tables 4 and 5 show that both SIM LC–MS [62] and tandem LC–MS–MS [63,80,81] techniques have been reported for urine and blood analysis. LC–MS–MS compared to LC–MS allows increased selectivity for phytoestrogen quantification because daughter ions arising from characteristic mass spectral fragmentation patterns are monitored. Tables 4 and 5 show that the limits of detection for the techniques are sensitive, though a poor limit of detection was reported for equol in the method described by Cimino et al. [62]. Recoveries are generally greater than 90% except those reported in Refs. [81,82]. In the method reported by Coward et al. [80], inter and intra-assay variation was below 10% for both daidzein and genistein but was higher for the metabolites dihydrodaidzein (DHD) and *O*-desmethylangolensin (DMA) in plasma (inter-assay=42–53 and 10–36% and intra-assay=26–45 and 10–16%, respectively). Valentín-Blasinj et al. [63] assessed inter-assay variation for serum and urine spiked with analytes. At low concentration in serum the inter-assay variation was below 10% except for equol and genistein (14 and 19%, respectively). In urine inter-assay variation was less than 10% except for matairesinol and coumestrol (13% and 14%, respectively). Inter- and intra-assay performance was assessed in the method of Cimino et al. [62]. Inter-assay variation was very high when free aglycones were measured as the levels of these were very close to analyte detection limits. Inter-assay coefficients for the analysis of glucuronide-conjugated isoflavonoids were between 10 and 17% except glycitein (29%). Intra-assay variation was less than 10%. These parameters were less than 10% for the method of Doerge et al. [81] though this is based on the report of Holder et al. [82] where the serum assessed originated from rats.

Immunoassay is one of the most sensitive analytical techniques to have been described for measurement of polyphenol phytoestrogens in human biological fluids (Tables 4 and 5). Detection limits for target analytes are below 1 nmol/l for most methods except for the ELISA for equol in plasma (3 nmol/l), the time resolved fluorescence immunoassay (TR-FIA) assay for daidzein in urine (2 nmol/l) and the ELISA for genistein (185 nmol/l). The average recovery of the listed methods is 92% and inter-/intra-assay variation is less than 10% for most methods.

A characteristic of antisera is ''cross-reactivity'' which is the ability of the antisera to bind with compounds of similar chemical structure to the target analyte. Of the immunoassay methods listed in Tables 4 and 5, some of the antisera cross-react better with isoflavonoids other than the target analyte. For instance in the TR-FIA described for genistein [65] the antisera cross-reacts fivefold stronger with biochanin A. However the results of the TR-FIA methods for daidzein, genistein and enterolactone show an excellent correlation (r^2= 0.96, 0.95 and 0.87 for genistein, daidzein and enterolactone, respectively) with those measured by ID-GC–MS-SIM for the same plasma samples [86]. In the same study correlation was also made with urine samples and though there was still very good correlation for daidzein, the TR-FIA assays for enterolactone and genistein were 30 and 115% higher, respectively, when compared with ID-GC–MS-SIM measurements. The higher values obtained by TR-FIA may be caused by isoflavone/lignan metabolites yet to be characterised for their cross reactivity, or ''non-specific'' interfering substances present in urine that vary in concentration from one individual to another. To overcome the high cross-reactivity associated with these assays, when applied to urine, raising antisera against an alternative isoflavone-protein immunogen, as has been proposed by the assay developers [65], may help or alternatively an isoflavone-europium detection tracer could be synthesized where the conjugation chemistry used to form the tracer differs from that used to form immunogen against which the anti-analyte serum is developed.

In general, as the full range of isoflavone and lignan metabolites become known it is important that possible cross-reactivity of these compounds in the described immunoassays is assessed.

2.4.1. Sample preparation

In human biological fluids ingested isoflavonoids and lignans together with their metabolites are generally conjugated with glucuronic acid or sulfate [1,2,62]. Tables 4 and 5 show that, to date, no method has been used to measure the conjugates and the aglycone form has always been analysed. To form the aglycones, enzymatic hydrolysis of the conjugates using β-glucuronidases and sulfatases is mostly used. A common source of these enzymes is from *Helix pomatia*. Purification of some commercial preparations is necessary because they have been found to be contaminated with low levels of polyphenol phytoestrogens [5]. Cleaner, but more expensive, sources of the deconjugation enzymes are available such as recombinant β-glucuronidases expressed in *Escherichia coli* or sulfatase from *Abalone entralis*. An overnight incubation of the urine or plasma/serum sample is commonly employed, though shorter incubation times, 0.5–3 h at 37°C, have been reported [62,81,91]. Following hydrolysis, purification of the aglycones from the aqueous buffered reaction medium by either solid-phase extraction, using C_{18} Sep-Pak cartridges [63], or solvent extraction with either diethyl ether or ethyl acetate are used [57,62,77,78,81,91] or a combination of solid-phase and organic solvent extraction [59,81]. The efficiency of the enzymatic hydrolysis of conjugated polyphenol phytoestrogens has been estimated by inclusion of glucuronide and sulfate conjugate internal standards such as 17-β-estradiol glucuronide [77–79,84], 4-methylumbelliferyl glucuronide or sulfate [63,80] and in the ID-GC–MS-SIM methods, tritiated estrone glucuronide and sulfate [54,55,68,69] or more recently tritiated estradiol-17-β-glucuronide [70]. This detail is omitted from Tables 4 and 5.

The GC–MS techniques that have been used for the analysis of human urine follow necessary sample preparation steps of varying complexity. The most complicated have been described by Adlercreutz and co-workers [54,69,70] for their ID-GC–MS-SIM method. Setchell and co-workers [5,7] and Morton and co-workers [71–76] describe less involved procedures. A simpler sample preparation procedure for urine analysis, prior to GC–MS-SIM has been described by Tekel et al. [56], where three different column chromatography steps are used (C_{18} solid-phase extraction cartridges, Chem Elut and Florisil, the last two columns connected in tandem) However the quoted recoveries were poorer than the complex method described by Adlercreutz et al. [54].

The simplest sample preparations are for immunoassay. Generally diluted samples are analysed following enzymatic hydrolysis of conjugates though, in the TR-FIA method described for daidzein [66], the enzymatic hydrolysis of sample is carried out within the microtitration plate wells and after 30 min incubation the rest of the immunoassay procedure is followed. Urine samples have also been analysed by TR-FIA directly following hydrolysis [65,85,86].

3. Method disadvantages/advantages

Table 6 summarises the main disadvantages and advantages of the principal methods used to analyse foods and human biological fluids for polyphenol phytoestrogens. For physico–chemical methods, the ability to measure a number of analytes in a single analysis is attractive compared with immunoassays, however a drawback to the analysis of phytoestrogens by GC–MS and LC–MS is that isotopically-labelled phytoestrogen conjugates for instance glycosides, glucuronides and sulfates are not available. Such compounds would be useful in GC–MS methods to help account for incomplete hydrolysis in the sample preparation stages. In LC–MS methods conjugate standards would aid analyte quantification. Non-isotopically labelled conjugate standards would also be of use in the analysis of polyphenol phytoestrogen by HPLC and immunoassay. These standards may soon become available as for instance a method for the chemical synthesis of daidzein-O^7-glucuronide has been reported [95].

Though immunoassays are most commonly used to measure a single analyte, they have the advantage in that a large number of samples can be measured in a single analysis. For non-isotopic immunoassays, microtitration plate formats are available in 96- or 384-well versions thus a large number of samples in replication can be measured in a single analysis. However, if key reagents are available, such as the various anti-analyte sera, more than one analyte in a sample may be measured over different microtitra-

Table 6
Summary of disadvantages and advantages of analytical methods for polyphenol phytoestrogen analysis

Method	Main	
	Disadvantages	Advantages
GC–MS	•Expensive instrumentation •Considerable expertise required for instrument operation •Complex, labour intensive sample preparation •Derivatization needed to produce volatile analytes •Only applicable to analysis of aglycones •Unsuitable for analysis of large sample numbers, e.g., epidemiological studies •Mass spectrometry not a technique available in all laboratories	•Sensitive (very low detection limits) and specific •Multi-analyte detection per assay •May be used for new metabolite discovery and identification
HPLC–UV and HPLC–DAD	•Less sensitive than GC–MS •Less specific than GC–MS •Slow sample throughput limits analysis of large sample numbers	•Simpler pre-analysis sample preparation than GC–MS •Multi-analyte detection per assay •Glycosides and aglycones can be measured •Most laboratories have HPLC
HPLC–coularray	•Expensive instrumentation and high purity solvents •Considerable expertise required for instrument operation	•Simple sample preparation •More sensitive and specific than UV or DAD •Wide analytical range •Multi-analyte detection per assay •Glycosides and aglycones can be measured
HPLC–MS	•Expensive instrumentation •Considerable expertise required for MS instrument operation •Mass spectrometry not a technique available in all laboratories	•Sensitive (very low detection limits) and specific •Multi-analyte detection per assay. Glycoside and aglycone analysis possible •Easy sample preparation •Higher sample throughput than other HPLC methods possible [74]
Immunoassay general irrespective of format	•Long time to generate key reagent (anti-analyte antibodies) •Full characterization of anti-analyte antibody specificity •Single analyte determination	•Very sensitive •Easy sample preparation •High throughput assays •Well suited technique for epidemiological and intervention studies
RIA	•Stability of radioactive labels •Disposal of radioactive waste	•Most sensitive of immunoassay formats
TR-FIA	•Expensive instrumentation	•Commercial assay kits available. No need for each laboratory to develop anti-analyte antibody •Highly reproducible
ELISA		•Inexpensive instrumentation

tion plates or within the same plate in the case of the 384-well format.

Immunoassays have been validated against other analytical techniques and excellent correlation has been demonstrated [86].

A significant disadvantage of immunoassay for small molecular mass compounds is the necessity to form an analyte–protein conjugate for generation of the key analytical reagent. The variety of conjugation chemistries that have been reported show that this is not a facile procedure [66,67,83,84,87,92–94] and very careful immunogen design is needed to produce antisera with high affinity for the target analyte and low cross-reactivity.

4. Suitability of analytical techniques to analysis problem

Very sensitive analytical techniques have been utilised for polyphenol phytoestrogen analysis and such techniques are necessary for the analysis of human biological fluids particularly serum as well as non-soy foods.

The most appropriate methods for the discovery and identification of unknown polyphenol phytoestrogens are GC–MS and LC–MS but these techniques are not the best for routine analytical situations because of their cost, though there are many reports in the literature where these analytical methods have been employed in a routine manner.

For the analysis of soy foods, analytical techniques of the utmost sensitivity are not really necessary because the isoflavonoid analytes are present in high concentrations. HPLC–DAD is an appropriate technique and newer reported methods show that the length of the chromatographic run can be reduced to less than 20 min with retention of adequate resolution [10]. This enhances the sample throughput of the technique but still does not offer the same throughput potential as immunoassay procedures.

HPLC with coularray detection is promising for sensitive measurement of multiple analytes with high resolution. This method potentially does not require the deconjugation of glucosides in food matrices or deconjugation of glucuronides and sulfates in human biological fluids. However the method is costly and has other disadvantages (Table 6). For routine measurements of human biological fluids in studies such as epidemiological screenings where very large sample numbers can be generated, immunoassay is very appropriate as it offers the advantage of high sensitivity with low cost analysis.

5. Conclusions

A variety of analytical techniques have been described for the analysis of polyphenol phytoestrogens in foods and human biological matrices. These techniques have been reported to be accurate and reproducible and in the case of GC–MS, LC–MS, HPLC–coularray and immunoassay very sensitive. HPLC–DAD is less sensitive but the continual development of instrumentation allowing improved resolution and sensitivity as well as the continuing development of new analytical techniques and refinements to existing techniques means this method is still useful. One under developed area that requires particular attention is the analysis of plant lignans.

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