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Analysis of phyto-oestrogens in biological matrices

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A review covering different methods for the analysis of phyto-oestrogens in biological matrices is presented. Sample pretreatment and analysis of isoflavonoids and lignans by HPLC and GC with various detection methods are discussed. The immunoassay method is also briefly presented.

Analysis of phyto-oestrogens: Isoflavonoids: Lignans

Introduction

Phyto-oestrogens, a group of plant polyphenols, have been shown to have both oestrogenic and anti-oestrogenic properties. Phyto-oestrogens along with their mammalian metabolic products have been isolated from various human body fluids. They are structurally similar to the endogenous hormone oestradiol (**1**; Fig. 1) and have been shown to bind to oestrogen receptors. This raises the possibility that they may be protective in hormone-related cancers. Clinical trials suggest that phyto-oestrogens may also lower the risk of other hormone-dependent diseases such as cardiovascular diseases and osteoporosis, together with menopausal symptoms. Many of the potential health benefits of phyto-oestrogens may be attributable to metabolic properties that do not involve oestrogen receptors, such as their influence on enzymes, protein synthesis, cell proliferation, angiogenesis, calcium transport, Na^+/K^+ adenosine triphosphatase, growth factor action, vascular smooth muscle cells, lipid oxidation and cell differentiation (Tham *et al.* 1998). In this connection it is significant that health claims of soya foods rich in isoflavonoids have received authorisation by the US Food and Drug Administration (Federal Register 64FR57699, 26 October 1999).

Phyto-oestrogens presented in this review are divided into three main classes: isoflavonoids, lignans and coumestans. Isoflavones, a subclass of the isoflavonoids, are found in a variety of plants, but predominantly in leguminous plants (Fabaceae) and especially in soya. In plants phyto-oestrogens occur mostly as various glycosides. The best-known non-steroidal phyto-oestrogenic isoflavones include daidzein (**2**), genistein (**3**), formononetin (**4**), biochanin A (**5**) and glycitein (**6**), along with a coumestan called coumestrol (**7**; Fig. 1). They exist in plants usually as β-glucoside conjugates, 6″-*O*-acetyl- and 6″-*O*-malonyl-β-glucosides. After ingestion, phyto-oestrogens undergo different metabolic reactions in man. First, they are hydrolysed by intestinal glucosidases to release the aglycones, which are then metabolised further. Genistein is reduced to dihydrogenistein (**8**), 6-hydroxy-*O*-desmethylangolensin (**9**) and ethylphenol (**10**; Fig. 2). Formononetin (**4**) is demethylated to daidzein (**2**), which is then reduced to dihydrodaidzein (**11**), 7,4′-dihydroxyisoflavan-4-ol (**12**) and to an isoflavan called equol (**13**) that is further ring-opened to *O*-desmethylangolensin (**14**; Fig. 3; Heinonen *et al.* 1999; Setchell & Cassidy, 1999). Aglycones and their metabolites are conjugated to glucuronides or sulphates and only a minority exists as free aglycones.

Lignans are present in a wide variety of plants. The highest amounts of lignans in the human diet have been detected from flaxseed. However, due to the relatively low consumption of flaxseed products, the most important sources of lignans are different varieties of cereal and whole-grain products, particularly rye and barley. Mammalian lignans, also called enterolignans, were first detected in human urine in 1979 (Setchell & Adlercreutz, 1979). Their definitive identification in 1981 proved their structures as enterolactone (**15**) and enterodiol (**16**; Fig. 4; Setchell *et al.* 1981). Other lignans called enterofuran (**17**) and 7′-hydroxyenterolactone (**18**; Fig. 4) were later tentatively identified from also human urine (Adlercreutz *et al.* 1995*a*; Liggins *et al.* 2000). Only a few precursors of mammalian lignans are known. The most important plant lignans found so far are matairesinol (**19**) and secoisolariciresinol (**20**; Fig. 5), which have also been isolated from human urine together with the mammalian lignan

Abbreviations: 6′-OH-dehydro-*O*-DMA, 6′-hydroxy-dehydro-*O*-desmethylangolensin; APCI, atmospheric pressure chemical ionisation; BSA, bovine serum albumin; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; CE, capillary electrophoresis; DEAE, diethylaminoethyl; ESI, electrospray ionisation; FID, flame ionisation detector; HN-APCI, heated nebuliser APCI; LC, liquid chromatography; QAE, quaternary amine; SIM, selected ion monitoring; SPE, solid-phase extraction; TBDMS, t-butyldimethylsilyl; TEAP, triethylaminohydroxypropyl; VENUS, Vegetal Estrogens in Nutrition and the Skeleton.

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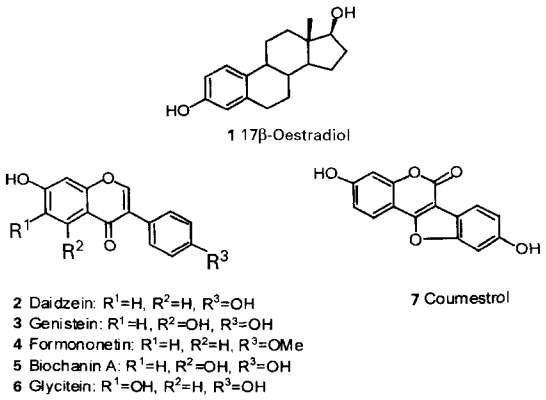

Fig. 1. 17β-Oestradiol (**1**) and common isoflavonoid phyto-oestrogens (**2**–**6**).

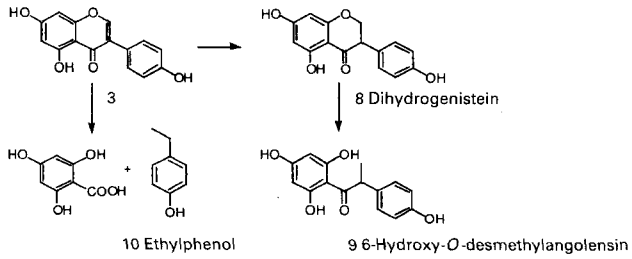

Fig. 2. Metabolism of genistein (**3**).

precursors 7′-hydroxymatairesinol (**21**), lariciresinol and isolariciresinol (Bannwart *et al.* 1989; Lampe *et al.* 1999; Nesbitt *et al.* 1999; Rowland *et al.* 2000).

We discuss here briefly the published methods of extraction and analysis of phyto-oestrogens from biological matrices. The tables give a detailed survey of the relevant literature.

HPLC analysis

The predominant method of analysis of phyto-oestrogens in general is HPLC (Table 1). The main advantage is its ease of use. Samples do not necessarily require time-consuming pretreatment and may, in principle, be injected into a system right after the extraction and analysed as such. The problem is to achieve adequate resolution and sensitivity since phyto-oestrogen levels are usually quite low.

Commonly, u.v. detectors or u.v. diode array detectors are used, but recently new methods for detecting analytes have been introduced. For example, coulometric electrode array detection has been utilised for quantification of the mammalian lignans enterolactone and enterodiol from human fluids (Gamache & Acworth, 1998; Nurmi & Adlercreutz, 1999). Coulometric array detection uses a porous flow-through graphite working electrode. It has a high surface area, which allows efficient electrolysis. This results in a highly reproducible total peak area (coulombs) and very low susceptibility to loss of signal from adsorptive effects. This technique has significant advantages in the detection of phenolic phytochemicals owing to the inherent sensitivity, selectivity and linear response range of electrochemical detectors. Also, because of the unique properties

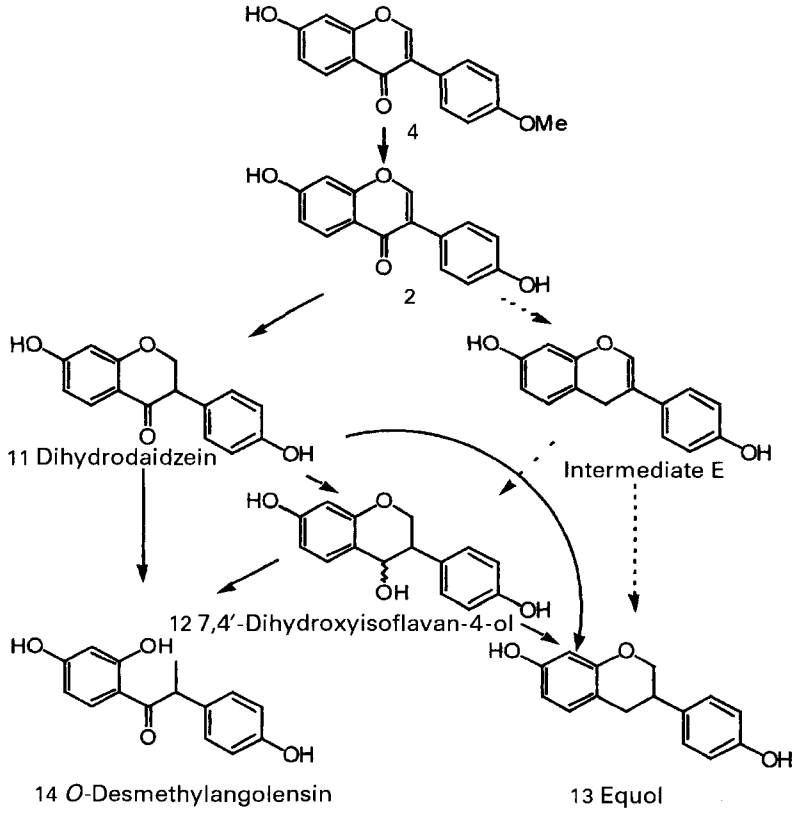

Fig. 3. Metabolism of formononetin (**4**) and daidzein (**2**). Intermediate E tentatively identified (Heinonen *et al.* 1999).

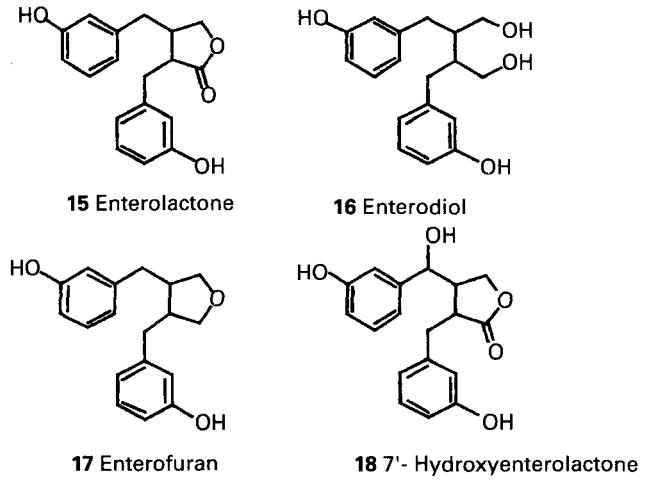

Fig. 4. Mammalian lignans.

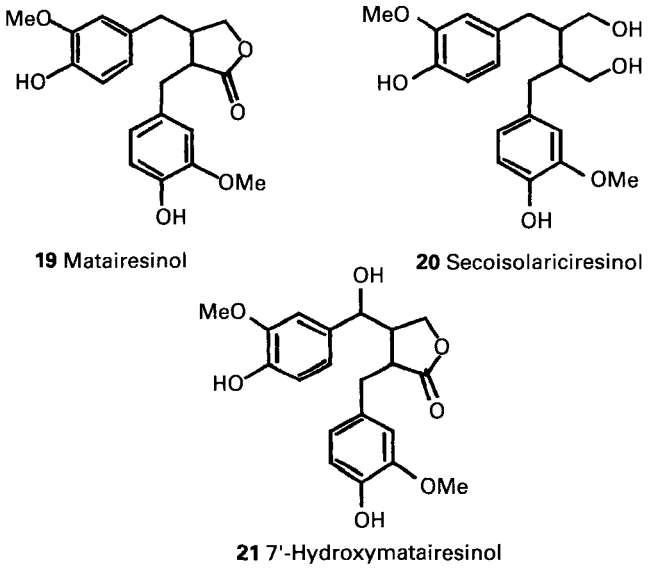

Fig. 5. Common plant lignans.

of the coulometric electrode, resolution of co-eluting solutes can be achieved on the basis of small differences in their oxidation–reduction behaviour (Gamache & Acworth, 1998).

The breakthrough in the application of HPLC–MS came from the development of two interfaces, the electrospray ionisation (ESI) interface and the heated nebuliser atmospheric pressure chemical ionisation (HN-APCI) interface (Barnes *et al.* 1998). HPLC–MS has the advantage over GC–MS in that it is not necessary to prepare volatile derivatives and that conjugated forms, such as the aglycones, can be analysed as easily. ESI-MS is well suited for the analysis of phyto-oestrogen conjugates such as sulphates and glucuronides in human fluids. Thermally labile conjugates form molecular ions in ESI. With the APCI interface, however, these physiological conjugates decompose since they are not stable enough. Instead of forming molecular ions, they form aglycone ions (Barnes *et al.* 1998).

HPLC with u.v./u.v. diode array detection

Franke *et al.* (1995) developed a method for the analysis of isoflavonoid phyto-oestrogens in human urine. In successive papers they extended the method for determinations in food and in human milk (Franke & Custer, 1996; Franke *et al.* 1998*a*,*b*), with a few changes to the original method. Daidzein, genistein, formononetin, biochanin A, coumestrol and their metabolites, such as equol and *O*-desmethylangolensin, were analysed by HPLC after isolation by solid-phase extraction (SPE; C-18). Samples were hydrolysed enzymatically by incubating for 24 h at 37°C, after being mixed thoroughly with a freshly prepared mixture of acetate buffer, ascorbic acid and glucuronidase/sulphatase. The samples were then centrifuged and the supernatants used for injection to HPLC. A reversed-phase column, coupled to a C-18 direct-connect guard column, was used. Gradient elution with a flow rate of 0·8 ml/min used two mobile phases (acetonitrile and then acetic acid–water, 10:90). Analytes were identified by their retention times, after a u.v. scan (fluorometric detection for coumestrol: excitation 340 nm, emission 418 nm). The detector used was a diode array (260 nm; 280 nm for equol and 342 nm for coumestrol elution). The detection limits were 5–780 nM (for 20 μl injection). For estimation of recovery, flavone was used as an internal standard.

Supko & Phillips (1995) reported a procedure involving isocratic reversed-phase HPLC with u.v. detection for the determination of genistein in biological matrices with very high recoveries (plasma mean: 94·8 %, urine mean: 91·4 %). The analyte was extracted with t-butylmethyl ether. The extract was centrifuged and supernatant recovered. After evaporation of the solvent, the residue was dissolved in a solution of methanol and ammonium acetate buffer (0·05 M, pH 4·5). An isocratic elution with a C-8 reversed-phase column was employed. The effluent was monitored at 260 nm. The lowest detectable concentration of genistein was 0·02–1·00 μg/ml with a sample volume of 50 μl. Simply increasing the sample size, without otherwise modifying the assay procedure, increased the sensitivity of detection. A ten-fold improvement was afforded by increasing the sample size to 250 μl.

Nose *et al.* (1992, 1993) used a reversed-phase HPLC method for analysis of the lignans arctiin, tracheloside and their metabolites in the rat gastrointestinal tract. Free lignans were extracted from serum with dichloromethane and from conjugated forms after hydrolysis. *p*-Hydroxybenzophenone was used as an internal standard. Analytes were detected with a u.v. detector at 280 nm.

HPLC with coulometric detection

Gamache & Acworth (1998) used HPLC with coulometric detection to analyse phyto-oestrogens in plasma, tissue and urine. Coulometric detection minimises sample pretreatment since it is sensitive, selective and also allows resolution of co-eluting analytes based on differences in their oxidation–reduction behaviour. A reversed-phase column (C-18, 150 mm×3 mm, 3 μm) was used together with a serial array of eight coulometric electrodes. The mobile phase consisted of sodium acetate with acetic acid, methanol

Table 1. Comparison of HPLC methods for the analysis of biological samples

Matrix	Analyte*	Pretreatment†	Recovery	Internal standard	Column	Mobile phase‡	Detector§	Detection limit	Reference
foods, urine, plasma, breast milk (human)	D, G, EQ, *O*-DMA, C	centrifugation, extraction with C-18 SPE or EtOAc (breast milk), enzymatic hydrolysis	85 % plasma, serum; 88–99 % breast milk	flavone	C-18 (10 mm×4·6 mm, 5 μm plus 150 mm×3·9 mm, 4 μm)	H_2O–MeOH–ACN–CH_2Cl_2–AcOH	u.v. DAD, 260/280 nm, FD, coulometric	26.6–164.2 nM (u.v.), 13.8–85.2 nM (ECD)	Franke *et al.* (1996, 1998*a*,*b*)
bile, urine, plasma (human)	D, G	centrifugation, enzymatic hydrolysis, extraction with diethyl ether		[^{14}C]genistein, BA	C-8 (150 mm×2·1 mm)	ACN–ammonium acetate, gradient/isocratic	ESI, APCI		Barnes *et al.* (1998)
plasma, urine, tissue (rat, man)	D, G, END, ENL, EQ	extraction with EtOH, centrifugation, enzymatic hydrolysis	90·23–96·05 %		C-18 (150 mm×3 mm, 3 μm)	ACN–MeOH–H_2O–AcOH–NaOAc	ECD	0·66–1·98 ng/ml	Gamache & Acworth (1998)
urine (human), legumes	D, G, C, F, BA, EQ, *O*-DMA	centrifugation, extraction with C-18 SPE, acid/enzymatic hydrolysis	97·5–101·5 %	flavone	C-18 (10 mm×4·6 mm, 5 μm plus 150 mm×3·9 mm, 4 μm)	ACN–H_2O–AcOH	u.v. DAD, 260, 280 and 342 nm	0·13–20·15 ng/ml (urine)	Franke *et al.* (1995)
serum (rat)	D, G	centrifugal filtering, automated on-line SPE	>80 %	daidzein-d_3, genistein-d_4	C-18 (150 mm×2 mm, 5 μm)	ACN–H_2O–HCOOH, isocratic	ESI-SIM	0·020 μM	Doerge *et al.* (2000)
blood (rat)	G, D	centrifugation, enzymatic hydrolysis, extraction with EtOAc	85 %	daidzein-d_3, genistein-d_4	C-18 (150 mm×2 mm, 3 μm)	ACN–H_2O–HCOOH, isocratic/gradient	ESI-SIM	5 nM (50 μl blood)	Holder *et al.* (1999)
blood, tissue (rat)	G	centrifugal filtering, automated on-line SPE (blood); homogenisation, enzymatic hydrolysis, extraction with MeOH, centrifugation, SPE (tissue)	79–89 % (serum); 40–78 % (tissue)	genistein-d_4	C-18 (150 mm×2 mm, 5 μm)	ACN–H_2O–HCOOH, gradient	ESI-SIM, MS–MS	0·04–0·09 pmol/mg (ESI-MS), 0·01–0·03 (MS–MS)	Chang *et al.* (2000)
urine (human)	D, G, BA, GL, DD, DG, EQ	extraction with diethyl ether, enzymatic hydrolysis	90·8 %		C-16 (250 mm×4·6 mm, 5 μm)	MeOH–ammonium acetate–TEA gradient	APCI	5 ng/ml, 200 ng/ml (EQ)	Cimino *et al.* (1999)
cancer cell lines	G, BA				C-8 (300 mm×4·6 mm); C-8 (100 mm×2·1 mm)	ACN–H_2O–TFA; ACN–H_2O–ammonium acetate	u.v. 262 nm; ESI		Peterson *et al.* (1998)
rat intestine	G	lyophilisation, centrifugation, extraction with EtOH or MeOH–H_2O, enzymatic hydrolysis	96·4–100 %	*p*-nitrophenol	C-18 (125 mm×2 mm, 3 μm)	H_2O–ACN–THF-HCOOH, isocratic	u.v. 262 nm		Andlauer *et al.* (2000*a*,*b*)

rats	SDG								Tou *et al.* (1998)
bile, urine (rat)	END, ENL				C-18				Niemeyer *et al.* (2000)
urine, plasma, breast milk (human)	D, G, GL, F, BA, EQ, *O*-DMA	centrifugation, extraction with C-18 SPE or EtOAc, enzymatic hydrolysis	breast milk (EtOAc): 88–99 %	flavone	C-18 (10 mm×4·6 mm, 5 μm plus 150 mm×3·9 mm, 4 μm)	H_2O–MeOH–ACN–CH_2Cl_2–AcOH	u.v. DAD		Franke *et al.* (1998*a*,*b*)
plasma, urine (mice)	G	extraction with TBME, centrifugation	94·8 % (plasma), 91·4 % (urine)	4-hydroxy-benzo-phenone	C-8 (150 mm×3·9 mm, 4 μm)	ACN–ammonium formate buffer, isocratic	u.v. 260 nm, TSP-MS		Supko & Phillips (1995)
blood (rat)	D, G EQ	enzymatic hydrolysis, centrifugation, extraction with MeOH–AcOH			C-18 (150 mm×4·6 mm, 5 μm)	H_2O–MeOH–AcOH–LiAc, isocratic	amperometric, ECD, u.v. DAD	0·05 μM (D), 0·03 μM (G)	Piskula *et al.* (1999), Piskula (2000), Yamakoshi *et al.* (2000)
plasma (human)	D, DD, *O*-DMA, G	extraction with C-18 SPE or diethyl ether, enzymatic hydrolysis	75·4–94·0 % (SPE), 84·2–94·6 % (ether)	BA	C-8 (100 mm×4·6 mm)	ACN–ammonium acetate, isocratic/ gradient	HN-APCI		Coward *et al.* (1996)
plasma (human)	D, G, Din, Gin, EQ, *O*-DMA, SECO, DD, DG, MAT, ENL, END, ENF	enzymatic hydrolysis, extraction with diethyl ether	68–91 %	[^{3}H]oestra-diol-17β-D-glucuronide	C-18 (150 mm×3 mm, 3 μm)	sodium acetate buffer–MeOH–ACN, gradient	ECD	3·4–40·3 pg on column	Nurmi & Adlercreutz (1999)
plasma, urine (rat)	D	centrifugation, extraction with EtOAc, enzymatic hydrolysis			C-18 (250 mm×4·6 mm, 5 μm)	sodium phosphate buffer–MeOH, gradient	u.v.		Yasuda & Oshawa (1998)
urine, faeces (human)	D, G				C-18 (300 mm×3·9 mm)		u.v. 254 nm		Xu *et al.* (2000*b*)

* Analyte: D, daidzein; G, genistein; EQ, equol; *O*-DMA, *O*-desmethylangolensin; C, coumestrol; END, enterodiol; ENL, enterolactone; F, formononetin; BA, biochanin A; GL, glycitein; DD, dihydrodaidzein; DG, dihydrogenistein; SDG, secoisolariciresinol diglycoside; Din, daidzein-7-*O*-glucoside; Gin, genistein-*O*-glucoside; SECO, secoisolariciresinol; MAT, matairesinol; ENF, enterofuran.
† Pretreatment: SPE, solid-phase extraction; EtOAc, ethyl acetate; EtOH, ethanol; MeOH, methanol; TBME, t-butylmethyl ether.
‡ Mobile phase: ACN, acetonitrile; CH_2Cl_2, dichloromethane; AcOH, acetic acid; NaOAc, sodium acetate; HCOOH, formic acid; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; LiAc, lithium acetate.
§ Detector: DAD, diode array detector; FD, fluorescence detector; ESI, electrospray ionisation; APCI, atmospheric pressure chemical ionisation; ECD, electrochemical detector; SIM, selected ion monitoring; TSP, thermospray; HN, heated nebuliser.

and acetonitrile. For the determination of free phyto-oestrogens in urine, samples were diluted with the mobile phase, centrifuged and analysed as such in runs of 30 min. To determine total phyto-oestrogens samples were first hydrolysed with β-glucuronidase. Analytes were quantified using standard mixtures treated in the same way as samples. Limits of detection for enterodiol and enterolactone were 10 pg.

A similar application to analyse plasma phyto-oestrogens with HPLC coulometric electrode array detection was developed by Nurmi & Adlercreutz (1999). Plasma samples were hydrolysed by glucuronidase/sulphatase treatment and extracted with diethyl ether. Evaporated samples were redissolved in methanol and analysed. Recoveries were determined with [^{3}H]oestradiol-17β-D-glucuronide. Separation was carried out using gradient elution with sodium acetate buffer, methanol and acetonitrile. A C-18 reversed-phase column (150 m × 3 mm, 3 μm) was used. Total analysis time was 85 min including stabilising time. Detection limits were 6·2 pg (enterolactone), 5·8 pg (enterodiol), 6·5 pg (matairesinol), 3·4 pg (secoisolariciresinol) and 5·4 pg (anhydrosecoisolariciresinol = enterofuran).

HPLC with MS detection

Coward and co-workers developed a method using reversed-phase HPLC–MS (Coward *et al.* 1996; Sfakianos *et al.* 1997). The method allows the construction of a mass/intensity map of several isoflavonoid metabolites in a single analysis of 20 min. The sample was first extracted with a C-18 cartridge and an internal standard was added. The sample was then analysed with HPLC–MS or hydrolysed enzymatically and extracted again with cartridges. Analyses of isoflavonoids and other phyto-oestrogens were carried out on a C-8 reversed-phase HPLC column using a linear gradient of 0–50 % acetonitrile in 10 nM-ammonium acetate (pH 6·5), over 10 min, at a flow rate of 1 ml/min. The solute was introduced into MS via the HN-APCI interface operating in either the positive or negative mode.

Doerge and colleagues developed a simple and sensitive liquid chromatography (LC)–ESI-MS method using deuterated internal standards (daidzein-d_3 and genistein-d_4) for the determination of daidzein and genistein and their conjugates in rat blood (Holder *et al.* 1999). Serum and plasma samples in acetonitrile were vortexed, sonicated and centrifuged to remove precipitated proteins. After selective hydrolysis, the aglycones were extracted into ethyl acetate. The samples were then evaporated, reconstituted in methanol and diluted with water before analysis by HPLC. Chromatography was performed with a C-18 column using isocratic elution. The detection limits for daidzein and genistein were approximately 5 nM (50 μl of rat blood). The recoveries of the analytes were approximately 85 %. Later the method was modified to eliminate laborious clean-up procedures (Chang *et al.* 2000; Doerge *et al.* 2000). A restricted-access chromatographic medium was used for automation of the analysis. Enzymatically hydrolysed samples were subjected to centrifugal filtering before loading onto the trap cartridge for sample clean-up. This procedure gave >80 % recoveries. Rat tissue was also analysed for the determination of tissue genistein distribution. Thawed tissue was homogenised, extracted with methanol by sonicating the suspension and hydrolysed enzymatically, whereby total genistein levels were determined. Lipids were extracted from fatty tissues into hexane. After centrifugation, the supernatants were loaded onto SPE cartridges before being subjected to LC–ESI-MS analysis. Limits of detection varied depending on the tissue matrix and were in the range of 0·04–0·09 pmol/mg (LC–ESI-MS) and 0·01–0·03 pmol/mg (MS–MS). Recoveries of genistein from tissues ranged from 40 to 78 %.

Cimino *et al.* (1999) developed an LC method for the separation of isoflavones and their metabolites from urine, establishing the concentrations with HPLC–APCI-MS. For the extraction from urine, ammonium acetate was added to each sample, the total isoflavones were extracted in diethyl ether, and the organic layers were evaporated to dryness with nitrogen. The free isoflavones were extracted directly from the sample. The sulphates and glucuronides were subjected to enzymatic hydrolysis by β-glucuronidase or sulphatase or both, when total content was analysed. After evaporation the solid sample was dissolved in the mobile phase, which consisted of two different solvent compositions. The column was a C-16 (25 m × 4·6 mm, 5 μm). Isoflavones were eluted with a linear gradient. Detection sensitivity of carbonyl-containing isoflavones was increased by the infusion of 15 % ammonium hydroxide at 0·14 ml/min to the eluate. Analytes were detected by MS using negative single ion monitoring. Detection limits for all analytes, except for equol, were 5 ng/ml of urine. The detection limit for equol was 200 ng/ml of urine.

Urinary lignans have also been analysed by HPLC–APCI-MS (Horn-Ross *et al.* 1997). Phyto-oestrogens were extracted from urine with C-18 cartridges and 4-methylumbelliferone glucuronide was added as an internal standard. After hydrolysis with glucuronidase/sulphatase, aglycones were recovered by SPE as above and subjected to HPLC–MS analysis. A C-8 reversed-phase column was used (4·6 m × 150 mm, 30 μm pore size). The solvent gradient was 0–50 % acetonitrile in ammonium acetate over 15 min. After chromatographic separation, the eluate stream was diluted with ammonium hydroxide and multiple reaction monitoring was carried out by selection of parent molecular ions and specific daughter ions formed by collision with argon–10 % nitrogen gas.

Piskula *et al.* (1999) studied the absorption of daidzein and genistein aglycones and glucosides in the rat. The determination of isoflavones in rat plasma was conducted by HPLC. After enzymatic hydrolysis, samples were extracted with methanol–acetic acid. After centrifugation, the supernatant was diluted with water and analysed using a C-18 (150 mm × 4·6 mm, 5 μm) column and an amperometric electrochemical detector. For the blood plasma analysis, the isolation procedure was somewhat more complicated. After hydrolysis, plasma was extracted three times with methanol and centrifuged. Supernatant was diluted with water and extracted with chloroform. After extraction, the methanol–water phase was evaporated and the residue was redissolved in methanol. The sample was then centrifuged at 4°C and supernatant was evaporated again. Next, the residue was dissolved in

water, sonicated and extracted with a C-18 cartridge. After further evaporation, the sample was dissolved in methanol and subjected to HPLC analysis. Again, a C-18 column was used with diode array detector.

GC analysis

The analysis of phyto-oestrogens from biological samples often requires pre-purification (Table 2). Samples are first extracted from the sample matrix by organic solvents such as diethyl ether or ethyl acetate. Also, C-18 cartridges have been used for SPE. For the enhancement of sensitivity, the removal of interfering constituents such as steroids is required. This can be done by ion exchange chromatography on Sephadex beads carrying diethylaminoethyl (DEAE) and quaternary amine (QAE) moieties. Moreover, various conjugates and aglycones can be separated with the same procedure. When GC is employed for the analysis, samples also require hydrolysis and derivatisation. Since sample pretreatment involves several steps, it is necessary to use internal standards to correct for losses. Internal standards should be chemically analogous to the samples being analysed so that they behave similarly during extractions and isolations. This is usually achieved by using different homologues or compounds carrying stable isotopic labels such as ^{2}H or ^{13}C. It should be noted that when the analytes are derivatised as their silyl ethers for GC–MS analysis, the combined effect of heavier, naturally occurring, stable isotopes of Si and C is such that intense peaks appear at $M+1$, $M+2$, etc. in the mass spectra. Thus the contribution of a single synthetically introduced isotopic label, either ^{2}H or ^{13}C, is almost lost. This is why we have developed methods for the synthesis of stable polydeuterated standards that contain a minimum of three ^{2}H atoms and in some cases up to eight ^{2}H atoms (Wähälä & Rasku, 1997; Rasku *et al.* 1999*a*,*b*; Salakka & Wähälä, 2000). The mass spectral peak to be used as reference in quantification will then be free from interference from the natural abundance ^{13}C and heavier Si atoms present in the trimethylsilyl ether derivatives.

To avoid the creeping of analytes, all glassware is usually silanilased before use by a 10% solution of dimethylchlorosilane in heptane (Liggins *et al.* 2000) or a 1% solution in toluene (Adlercreutz *et al.* 1993).

Phyto-oestrogens are present in biological fluids usually as the glucuronide and/or sulphate derivatives. Adlercreutz *et al.* (1995*b*) have used a β-glucuronidase solution from *Escherichia coli* K12 in acetate buffer to hydrolyse glucuronides. Sulphates were solvolysed in dimethylformamide–6 M-hydrochloric acid–dichloromethane solution overnight at 37°C. Samples can also be hydrolysed with β-glucuronidase/sulphatase enzyme juice extracted from digestive juice and hepatopancreas of the snail *Helix pomatia*. Because the juice also contains small amounts of lignans and isoflavonoids, it has to be purified before use. Mazur *et al.* (1996) used 1% charcoal in 0·66 M-acetate buffer shaken overnight at room temperature. SPE C-18 cartridges (Setchell *et al.* 2001) and ion exchange columns (Morton *et al.* 1994) have also been used for purification. Samples are first hydrolysed if the total phyto-oestrogen content is to be analysed. Another way is to separate aglycones and conjugates from each other, or even different conjugates from each other, before the hydrolysis.

Trimethylsilyl derivatives are prepared either by pyridine–hexamethyldisilazane–trimethylchlorosilane (9:3:1) or *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). For the t-butyldimethylsilyl (TBDMS) ethers, *N*-methyl-*N*-TBDMS-trifluoroacetamide and TBDMS chloride in acetonitrile are used (Setchell *et al.* 2001). Being a strong silylating agent, BSTFA may cause the formation of artifacts (Wähälä *et al.* 1997, 1998; Heinonen *et al.* 1999). In addition to silylation of free hydroxyl groups, carbonyl groups may be silylated in the enol form. Furthermore, derivatisation of the isoflavone metabolites dihydrodaidzein and dihydrogenistein in dry pyridine may yield C-ring cleavage products referred to as dehydro-*O*-desmethylangolensin and 6′-hydroxy-dehydro-*O*-desmethylangolensin (6′-OH-dehydro-*O*-DMA), respectively. Derivatisation of dihydrogenistein with BSTFA produced 6′-OH-dehydro-*O*-DMA only as a minor product (Heinonen *et al.* 1999).

Isotope dilution GC–MS with selected ion monitoring

Adlercreutz *et al.* described a method for the quantitative determination of phyto-oestrogens in biological samples. They used isotope dilution GC–MS with selected ion monitoring (SIM). Stable deuterated derivatives of the compounds to be analysed are added to the sample at the start of the procedure. Recoveries are calculated by monitoring the mass peaks of analytes and co-eluting deuterated samples. The lignans enterolactone and enterodiol and the isoflavonoids daidzein, equol and *O*-desmethylangolensin were first analysed from human (Adlercreutz *et al.* 1986*a*) and chimpanzee urine (Adlercreutz *et al.* 1986*b*) using different modifications of the method. A slightly different method, developed originally for determining the whole oestrogen profile in urine, has been applied for the analysis of phyto-oestrogens in urine (Adlercreutz *et al.* 1991*a*,*b*) and plasma (Adlercreutz *et al.* 1993, 1994). Plasma samples were first extracted with C-18 SPE cartridges before ion exchange chromatography and, after evaporation of the solvent, were solvolysed with HCl. Free and conjugated phyto-oestrogens were separated with DEAE-Sephadex in the acetate form. The phyto-oestrogen fraction originally contained free and mono- and disulphated (now unconjugated after solvolysis) phyto-oestrogens. The fractions eluting subsequently contained mono- and diglucuronides and solvolysed sulphoglucuronides. This fraction was purified further with C-18 cartridges. Deuterated internal standards were then added to both samples and the conjugated phyto-oestrogen fraction was hydrolysed with β-glucuronidase. Owing to the lack of conjugated internal standards, the recoveries until this step were corrected using radioactive standards added at the beginning of the whole procedure. The standards used for this purpose were [^{2}H]oestrone sulphate and [^{3}H]oestrone glucuronide. Free and conjugated fractions were then subjected to QAE-Sephadex chromatography in the acetate form. The first fraction contained all oestrogens and enterolactone, enterodiol, matairesinol and equol. The second fraction contained *O*-desmethylangolensin, daidzein and genistein. Oestrogens from the

Table 2. Comparison of GC methods for the analysis of biological samples

Matrix	Analyte*	Pretreatment†	Internal standards	Recovery	Column	Detector‡	Detection limit	Reference
faeces (human)	MAT, ENL, END, D, EQ, *O*-DMA, G	extractionwithEtOH, centrifugation,extractionwithSPE, ionexchangechromatography	deuterated standards of corresponding analytes	97·0 % (mean)	12·5 m × 0·2 mm, 0·25 μm, 100 % polysiloxane	MS	1–4 nmol/24 h	Adlercreutz *et al.* (1995*b*)
urine (human)	D, G, EQ, DD, *O*-DMA	enzymatic hydrolysis, extraction with diethyl ether, Sephadex LH-20 chromatography	oestriol	82–93 %	30 m × 0·25 mm, 100 % polysiloxane	FID, MS		Kelly *et al.* (1993)
urine (human)	D, G, GL, DG, DD, ENL	enzymatic hydrolysis, extraction with diethyl ether, Sephadex LH-20 chromatography	oestriol, [^{3}H]oestradiol glucuronide	82–93 %	30 m × 0·25 mm, 100 % polysiloxane	MS		Joannou *et al.* (1995)
urine (human)	G, D, DD, *O*-DMA, EQ, GL, C, END, ENL, MAT	SPE extraction, enzymatic hydrolysis, ion exchange chromatography	deuterated standards of corresponding analytes			MS		Xu *et al.* (1998)
urine (human)	D, G, *O*-DMA, END, ENL, MAT	SPE extraction, ion exchange chromatography, enzymatic hydrolysis	deuterated standards of corresponding analytes			MS		Lampe *et al.* (1999)
foods, plasma (human)	SECO, MAT, END, ENL				15 m × 0·25 mm, 1 μm, 100 % polysiloxane	MS		Liggins *et al.* (2000)
urine (human)	D, G, F, BA	centrifugation, enzymatic hydrolysis, SPE extraction	6-hydroxyflavone	63·5–89·6 % (free), 56·5–77·1 % (conjugated)	30 m × 0·25 mm, 0·25 μm, (5 % phenyl)-methylpolysiloxane	MS	1·05–2·3 ng/ml	Tekel *et al.* (1999)
urine (human)	D, G, EQ	enzymatic hydrolysis, SPE extraction	4′,7-dihydroxyflavone	84·5–91·8 %	30 m × 0·53 mm, 0·88 μm, 100 % polysiloxane	FID		Lu *et al.* (1995*a*,*b*, 1996)
plasma, prostatic fluid (human)	D, G, EQ, ENL, END	enzymatic hydrolysis, extraction with diethyl ether, ion exchange chromatography	deuterated standards of corresponding analytes		30 m × 0·32 mm, 0·25 μm, 100 % polysiloxane	MS		Morton *et al.* (1994), Xu *et al.* (2000*a*)
plasma, prostatic fluid (human)	D, G, EQ, ENL, END	enzymatic hydrolysis, Sephadex LH-20 chromatography	deuterated standards of corresponding analytes		30 m × 0·32 mm, 0·25 μm, 100 % polysiloxane; 12 m × 0·32 mm, 1·0 μm	MS		Morton *et al.* (1997*a*,*b*, 1999)
in vitro fermentation	ENL, END	extraction with SPE, enzymatic hydrolysis, ion exchange chromatography	5α-androstron-3β, 17β-diol, stigmasterol		(5 % phenyl)(1 % vinyl)-methylpolysiloxane	FID		Thompson *et al.* (1991)

* Analyte: MAT, matairesinol; ENL, enterolactone; END, enterodiol; D, daidzein; EQ, equol; *O*-DMA, *O*-desmethylangolensin; G, genistein; DD, dihydrodaidzein; GL, glycitein; DG, dihydrogenistein; C, coumestrol; SECO, secoisolariciresinol; F, formononetin; BA, biochanin A.
† Pretreatment: EtOH, ethanol; SPE, solid-phase extraction.
‡ Detector: FID, flame ionisation detector.

first fraction were further separated with QAE-Sephadex in the carbonate form, followed by derivatisation and GC–MS-SIM analysis. The method was modified for the analysis of phyto-oestrogens in plasma (Adlercreutz *et al.* 1993). By this method 15 oestrogenic compounds (endogenous oestrogens and phyto-oestrogens) can analysed by one run. This method was utilised by Lampe and co-workers to compare the effect of vegetable, fruit and legume consumption on urinary phyto-oestrogen isoflavonoid and lignan excretion (Hutchins *et al.* 1995; Kirkman *et al.* 1995; Slavin *et al.* 1998; Lampe *et al.* 1999, 2001).

The method has been developed further to determine the pattern of conjugation of the phyto-oestrogens in human urine (Adlercreutz *et al.* 1995*a*). The first DEAE-Sephadex chromatography in the acetate form is modified to separate the free fraction, mono- and disulphate as well as mono-, di- and sulphoglucuronide fractions. The method has been applied for the determination of unconjugated lignans and isoflavonoids in human faeces (Adlercreutz *et al.* 1995*b*). Jacobs *et al.* (1999) have modified the method for determining metabolites of mammalian lignans in human urine and further to determine metabolites of enterolactone and enterodiol in rat bile and urine (Niemeyer *et al.* 2000). The method has also been applied by Xu's group to determine phyto-oestrogens in human urine (Xu *et al.* 1998, 2000*a*,*b*; Duncan *et al.* 2000).

GC–MS

Kelly and associates described a simple method for determining total isoflavones and their metabolites together with enterolactone in human urine (Kelly *et al.* 1993; Joannou *et al.* 1995). Urine samples were first hydrolysed with β-glucuronidase aryl sulphatase. The samples were then extracted with diethyl ether, the organic phase evaporated and the residue dissolved in ethanol. The analytes were isolated by partition chromatography on Sephadex LH-20 and derivatised prior to analysis with GC and GC–MS. [^{3}H]oestradiol glucuronide and oestradiol were used as internal standards. Derivatisation by BSTFA in this method was proved later to have produced metabolite artifacts (Wähälä *et al.* 1997, 1998; Heinonen *et al.* 1999).

Setchell *et al.* (1997, 2001) reported a method for determining the isoflavones daidzein, genistein, glycitein, biochanin A and formononetin from human plasma and urine using two stable, isotopically labelled internal standards and an isoflavone homologue. Total and free isoflavones were identified separately. First, [^{13}C]daidzein, [^{13}C]genistein and 4′,7-dihydroxyflavone were added as internal standards to plasma samples. For total isoflavones, samples were extracted with C-18 BondElut cartridges. Samples were then hydrolysed with β-glucuronidase/sulphatase from *H. pomatia*. Hydrolysed samples were extracted again with C-18 BondElut cartridges. Isoflavones were separated from neutral compounds, purified with triethylaminohydroxypropyl(TEAP)-Sephadex LH-20 in the hydroxide form and derivatised. Free isoflavones were determined as above but without the hydrolysis step. TBDMS-derivatised samples were analysed with GC–MS.

Morton *et al.* (1994, 1997*a*) have described a GC–MS method for determining the total phyto-oestrogens enterodiol, enterolactone, daidzein, genistein and equol in human plasma. A plasma sample was hydrolysed with β-glucuronidase and deuterated internal standards were added. The hydrolysate was extracted with diethyl ether and the organic phase was collected. Phyto-oestrogens were isolated with TEAP-Sephadex LH-20 in the hydroxide form as described by Setchell *et al.* (1976). BSTFA-derivatised samples were then analysed with GC–MS using single ion recording. Later the method was modified for the analysis of prostatic fluid and blood (Morton *et al.* 1997*b*). Phyto-oestrogens were isolated with Sephadex LH-20 as described by Kelly *et al.* (1993). For serum analysis, ethyl acetate was used for extraction (Morton *et al.* 1999).

Liggins *et al.* (2000) identified a lignan metabolite called enterofuran from urine by a simple GC–MS method. The urine sample was hydrolysed with β-glucuronidase and phyto-oestrogen aglycones were extracted with ethyl acetate. After evaporation of the solvent, the sample was derivatised and analysed with GC–MS. Similarly, a simple GC method utilising a flame ionisation detector (FID) was employed for total and unconjugated isoflavones in human urine by Lu *et al.* (1995*a*,*b*, 1996). Samples were hydrolysed with β-glucuronidase/sulphatase and aglycones were isolated by chromatography.

Thompson *et al.* (1991) analysed mammalian lignans after *in vitro* fermentation with colonic microflora. Enterodiol and enterolactone were extracted with C-18 cartridges and hydrolysed with β-glucuronidase. Aglycones were then extracted again with C-18 cartridges, further isolated and purified with DEAE-Sephadex in the hydroxide form, and analysed after derivatisation by GC-FID. The method was subsequently applied to the determination of lignan metabolites in rat urine (Serraino & Thompson, 1992; Thompson *et al.* 1996) and later modified further by using a mass detector instead of FID (Jenab & Thompson, 1996; Rickard *et al.* 1996). Deuterated standards of enterolactone and enterodiol were used.

Immunoassays

The methods described above are not suitable for screening purposes in large populations. In addition, they are not sensitive enough for the assay of unconjugated phyto-oestrogens in plasma. These disadvantages have led to the application of a new analytical method based on immunoassay.

Radioimmunoassay

A radioimmunoassay for the determination of formononetin was established by Wang (1998), employing rabbit antisera against formononetin-7-bovine serum albumin (BSA) and the ^{3}H-labelled, homologous, radioligand conjugate formononetin-7-([^{3}H]leucine). This radioimmunoassay procedure enabled the quantification of 200 pg/mg of plasma or 50 pg/mg of mammary tissue. Lapcík *et al.* (1997) have developed a radioimmunoassay procedure to determine daidzein in plasma, serum and urine using daidzein-4-BSA (Lapcík *et al.* 1997; Al-Maharik *et al.* 1999, 2000) for immunisation and an ^{125}I-labelled tracer. Daidzein was measured either directly or after extraction in ether. The working range of the assay was 1·5–200 pg/tube. The method did not

discriminate between daidzein and its 4′-methoxy derivative formononetin, which caused almost 60% cross-reaction. This is because the antisera prepared via the 4′-position also recognise 4′-methoxy derivatives. In the same way, antisera prepared via the 7-position recognise 7-methoxy derivatives (Lapcík *et al.* 1999). Moreover, cross-reaction with 4′-sulphates and β-glucuronides probably occurred since daidzein values after extraction were only 8% of those obtained with direct serum analysis. Recently, Lapcík *et al.* (1998) established two radioimmunoassays for genistein based on polyclonal antibodies against genistein-4-*O*-(carboxymethyl) ether-BSA and against genistein-7-*O*-(carboxymethyl) ether-BSA conjugates (Lapcík *et al.* 1998; Al-Maharik *et al.* 1999, 2000). The minimum detectable limits for the assays were 1·2 and 2·8 pg/tube, respectively.

Time-resolved fluoroimmunoassay was developed for the rapid analysis of enterolactone in human plasma, using europium chelate as a label, by Adlercreutz *et al.* (1998). The method used 5′-*O*-carboxymethoxyenterolactone haptens (Mäkelä *et al.* 2000) for the immunisation and production of tracer. No cross-reactions were observed for phyto-oestrogens except to a small extent with enterodiol (0·28%). The minimal amount of enterolactone distinguishable was 2·1 pg/20 μl. The rapid method allowed analysis of 100 samples in 4 h (Adlercreutz *et al.* 1998), with a ten- to 100-fold increase in sensitivity and assay range compared with conventional enzyme immunoassay and fluoroimmunoassay methods. Because values for plasma enterolactone were found to be overestimated, the method was modified by lowering the amount of hydrolysis reagent and thus reducing the absolute amount of sulphatase, which caused the error in the values (Stumpf *et al.* 2000). Later, a method for determining daidzein and genistein in plasma and urine was developed (Uehara *et al.* 2000; Wang *et al.* 2000). Phyto-oestrogens were measured from hydrolysed samples. The minimal amounts of daidzein and genistein, distinguishable from zero sample, were 1·8 pg/20 μl and 3·2 pg/20 μl, respectively. Kohen *et al.* (1998) reported a highly specific time-resolved fluoroimmunoassay method for the measurement of daidzein in urine. Daidzein was analysed in urine after hydrolysis with β-glucuronidase.

ELISA was also developed for the analysis of formononetin, daidzein, equol, biochanin A and genistein (Bennetau-Pelissero *et al.* 2000; Le Houérou *et al.* 2000). Table 3 summarises some immunoassay methods for the quantification of phyto-oestrogens.

Recent advances

A simple and rapid method for the analysis of isoflavones in food samples has been developed by Wang & Sporns (2000). They used the matrix-assisted laser desorption ionisation–time-of-flight technique to detect daidzein, genistein and glycitein, together with their various conjugates, in soya products. By selecting the proper matrix, this technique provides the isoflavone profile of a food sample in a few minutes and can be used for both quantitative and qualitative analysis especially in conjunction with other techniques such as HPLC.

Capillary electrophoresis (CE) is powerful technique, affording rapid, high-resolution separations (10^4–10^6 theoretical plates) while requiring just femtomoles of sample. The utility is greatly enhanced by MS detection, particularly with ESI, which can be used to produce ions from thermally labile, non-volatile compounds such as conjugated phyto-oestrogens. It has been used to determine isoflavones from soyabean seeds using u.v. detection (Aussenac *et al.* 1998). A CE–MS study of isoflavones has also been published (Aramendia *et al.* 1995). One potential limitation with CE concerns the low sample loads owing to the small inside diameter of the capillaries, the sample injection volumes being confined to the low nanolitre and low picomole range in order to maintain high separation efficiency. Hence the concentration detection limit for CE–MS is often unsatisfactory (Aramendia *et al.* 1995).

Conclusions

Quantitative analyses of isoflavonoids in plants, foods and biological fluids are important in epidemiological, pharmacological, phyto-oestrogenic, chemotaxonomic, breeding and other biochemical studies. Isoflavonoids are present in plants as mixtures of aglycones and glycosides, and in biological fluids as mixtures of aglycones with glucuronides, sulphates or with sulphates and glucuronides together. In addition, initial extracts may contain many structurally related compounds. These include flavonoids, steroids and other polyphenolic compounds. Methods for quantification

Table 3. Comparison of immunoassay methods for the analysis of biological samples

Matrix	Analyte*	Detection limit	Method†	Reference
serum, urine (human)	D	0·4 pg/tube	RIA	Lapcík *et al.* (1997)
serum (human)	G	1·2 pg/400 μl	RIA	Lapcík *et al.* (1998)
plasma (murine), mammary glandular tissue	F	4 ng/ml (plasma), 50 pg/mg (glandular tissue)	RIA	Wang (1998)
	D, G, EQ	1·3–7·5 pg/tube	RIA	Lapcík *et al.* (1999)
plasma (human)	ENL	2·1 pg/20 μl	TR-FIA	Adlerceutz *et al.* (1998)
urine (human)	D	0·5 ng/ml	TR-FIA	Kohen *et al.* (1998)
plasma (human)	D, G	1·8 pg/20 μl (D), 3·1 pg/20 μl (G)	TR-FIA	Wang *et al.* (2000)
urine (human)	D, G, ENL	2 pg/20 μl	TR-FIA	Uehara *et al.* (2000)
	F, D, EQ, BA, G		ELISA	Bennetau-Pelissero *et al.* (2000)

* Analyte: D, daidzein; G, genistein; F, formononetin; EQ, equol; ENL, enterolactone; BA, biochanin A.
† Method: RIA, radioimmunoassay; TR-FIA, time-resolved fluoroimmunoassay.

must therefore be capable not only of separating isoflavonoids from other compounds, but also of distinguishing between the various isoflavonoid structures. Phyto-oestrogens are presently identified and determined mainly by GC–MS and HPLC, techniques that are time-consuming and labour-intensive and require sophisticated and expensive instrumentation. More effective and economical methods for the monitoring of human exposure to phyto-oestrogens should be specific and sensitive and allow large numbers of samples to be processed reliably and rapidly. Immunoassay offers the advantages of speed, sensitivity and high throughput, with the potential for automation.

GC combines the advantage of high separation capability with the disadvantages of the need for pre-purification, fractionation, hydrolysis and derivatisation of samples. GC–MS is the basis of the majority of existing methods for the quantitative analysis of isoflavones and their metabolites in biological fluids, including urine, plasma and faeces. GC–MS in the SIM mode employs deuterated internal standards to compensate for losses during the isolation process.

HPLC methods have been developed to allow the determination of a variety of isoflavonoids, including aglycones and conjugated isoflavonoids. Use of fluorescence detection and photodiode array detection in place of u.v. detection is a useful way of increasing the sensitivity. Whilst u.v. photometry at 260 nm can detect nanogram quantities of daidzein and genistein, the limit of electrochemical detection is about 10 pg. Advantages of HPLC–MS over GC–MS analysis include higher precision, less manipulation, and applicability to non-volatile components with direct injection of the liquid sample.

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