This paper has been prepared for discussion by the COT Working Group on Phytoestrogens and does not necessarily reflect the final views of the Group.

PEG/2000/06

COMMITTEE ON TOXICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

WORKING GROUP ON PHYTOESTROGENS

CHEMISTRY OF PHYTOESTROGENS AND OVERVIEW OF ANALYTICAL METHODOLOGY

Introduction

This paper provides an overview of the chemical classification of dietary phytoestrogens and reviews the developments of the methodology used in the analysis of phytoestrogens.

Member's are invited to comment on PEG/2000/06.

In addition, member's comments are invited on the following specific questions

1. Members are asked to comment on the adequacy of the available analytical methodology.

2. Is it necessary to continue to gather information on the chemical forms of phytoestrogens as they occur in food?

3. Should research into the synthesis of labelled standards continue to be funded?

4. Is there a need for more information on the estrogenic potency of lignans and their prevalence in the UK diet?

5. The need to widen the analysis of phytoestrogens in food to include compounds such as the prenylated isoflavonoids?

6. Is LC-MS preferable as an analytical technique in the analysis of dietary phytoestrogens?

Chemistry of Phytoestrogens & Review of Analytical Methodology

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Chemistry of Phytoestrogens

Introduction

1. Phytoestrogens are chemicals of plant origin that have the ability to cause estrogenic and/or anti-estrogenic effects (Setchell & Cassidy, 1999). The biological function of these compounds in plants is uncertain however, it has been suggested that they may have antifungal, antibacterial or antiviral properties and maybe involved in signalling between nitrogen-fixing bacteria and leguminous plants (Mazur & Adlercreutz, 1998).

Animal studies

2. In animals, studies have shown that components present in plants and foods can elicit estrogenic effects. The fertility of some animals has been affected by components of the animal diet.

3. The phytoestrogen, formononetin, present in red clover was identified as the active agent which gave rise to “clover disease”, an infertility of ewes grazing on pastures rich in this plant (Shutt, 1976). The phytoestrogens, daidzein and genistein were responsible for the infertility of some captive cheetahs fed a soyabean enriched diet subsequently found to contain high quantities of these compounds (Setchell *et al.*, 1987).

Phytoestrogen classes

Table 1: Classes of estrogenic flavonoids and lignans.

Class	**Compound**	**Estrogenic activity** (relative to estradiol = 1)[a]
Isoflavones	biochanin A	1.7×10^{-4}
	daidzein	
	formononetin	2.2×10^{-5}
	genistein	5×10^{-4}
Coumestans	coumestrol	0.1
	methoxycoumestrol	
Flavones	apigenin	2.3×10^{-5}
	luteolin	
	flavone	2.2×10^{-5}

Flavonols	kaempferol quercetin	4.5×10^{-5}
Flavanones	naringenin dihydroxyflavenone	2.2×10^{-5}
Chalcones	phloretin isoliquiritigenin	3.3×10^{-5}
Lignans	matairesinol secoisolariciresinol	

[a] taken from Collins *et al.* (1997) utilising a recombinant human estrogen receptor reporter system in yeast.

4. Many natural substances present in plants have been found to exert estrogenic effects. The majority are phenolic compounds such as the flavonoids. Flavonoids are present in nearly all plants and can comprise up to 7% of the dry weight (Kuhnau, 1976).

5. There are several groups of flavonoids with estrogenic properties. Of these, the **coumestan** and **isoflavone** classes possess the greatest estrogenic activity (Collins *et al.*, 1997) (see table 1). A class of prenylated flavanones with estrogenic activities intermediate to those of the coumestans and isoflavones has recently been identified (Milligan *et al.*, 1999). **Lignans** have also been shown to exert estrogenic effects (Setchell & Adlercreutz, 1988).

Isoflavones

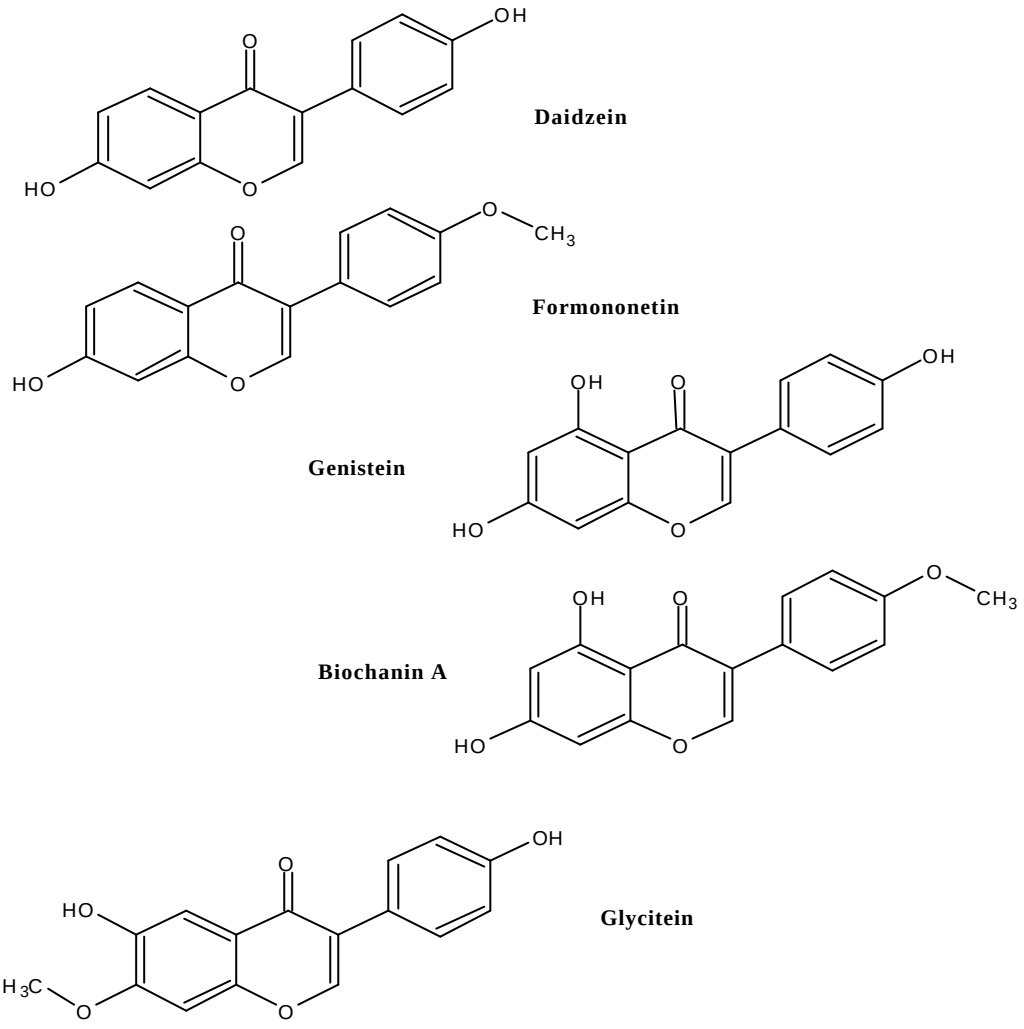
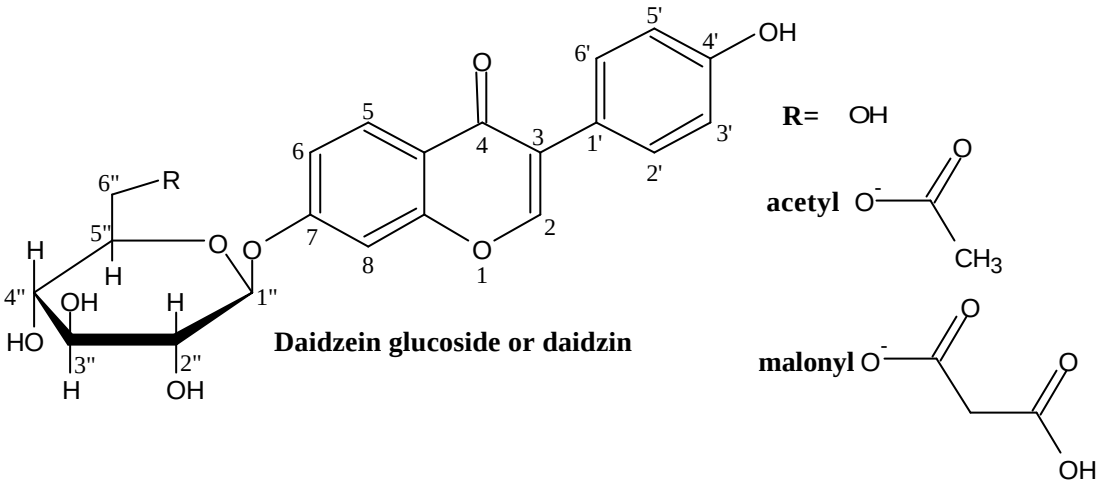


Figure 1: The isoflavone aglucones: genistein, biochanin A, daidzein, formononetin and glycitein.

6. The isoflavones are predominantly found in leguminous plants. **Genistein**, **daidzein**, **glycitein**, **biochanin A** and **formononetin** (see figure 1) are the most prevalent of the isoflavones identified (Bingham *et al.*, 1998).

Isoflavone structure

7. Genistein, daidzein and glycitein are commonly present in plants and foodstuffs as glucose conjugates (Bingham *et al.*, 1998), which are referred to as glucosides or glycosides. The glucosyl group is conjugated, via an ester bond, at the 7-isoflavone position and the sugar is often esterified with acetyl- or malonyl-groups at the 6”-position (see figure 2). 7-Glucosylgenistein is known as **genistin**, 7-glucosyldaidzein is known as **daidzin** and 7-glucosylglycitein is known as **glycitin**. When the sugar bears acetyl or



malonyl groups these terms are prefixed with 6”-acetyl or 6”-malonyl.

Figure 2: Isoflavone glucoside, acetylglucoside and malonylglucoside structures and numbering system.

8. Biochanin A and formononetin are methylated derivatives of genistein and daidzein. Biochanin A and formononetin can also occur as glucosides, which are known as ononin and sissotrin, respectively. Little information is available on the prevalence of these particular isoflavones in plants and foodstuffs.

9. Dietary isoflavones can be divided into four categories:
(a) **aglucones**: daidzein, genistein, glycitein, formononetin and biochanin A.
(b) **glucosides** or **glucones**: daidzin, genistin, glycitin, ononin and sissotrin.
(c) **acetylglucosides** or **acetylglucones**: 6”-acetyldaidzin, 6”-acetylgenistin and 6”-acetylglycitin.
(d) **malonylglucosides** or **malonylglucones**: 6”-malonyldaidzin, 6”-malonylgenistin and 6”-malonylglycitin.

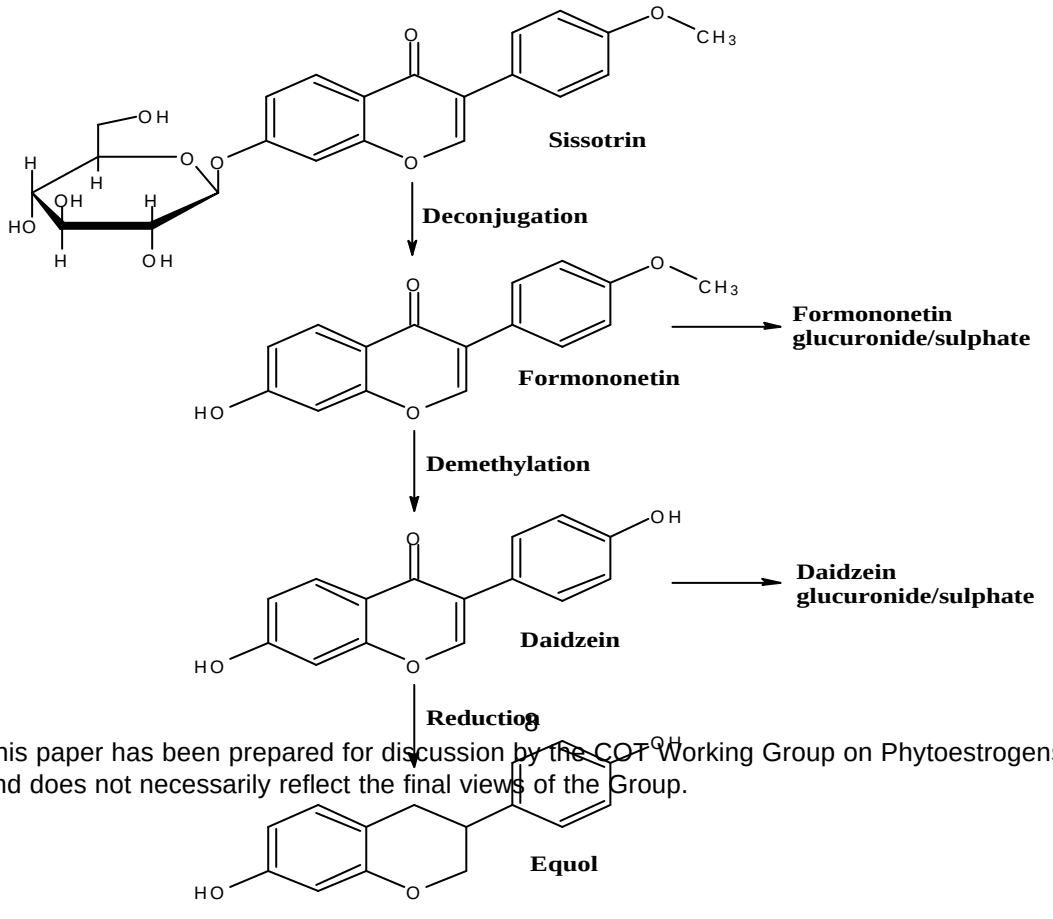
Physical & chemical properties of isoflavones

10. The isoflavone aglucones are stable under physiological conditions. However, the acetyl- and malonyl-glucose ester bonds are labile at elevated temperatures and under acidic or basic conditions. The glucose-isoflavone ether bonds are stronger but can break under acidic conditions and/or high temperatures.

11. The aqueous solubilities of the isoflavone aglucones are low and due to the acidic nature of the phenolic groups are pH dependent. Conjugation to glucose residues increases the solubility, while acetylation or malonylation of the glucones reduces solubility (Setchell & Cassidy, 1999). The methylated derivatives, biochanin A and formononetin are less soluble than genistein and daidzein, respectively.

Metabolism of isoflavones

12. It is generally thought that the conjugated isoflavones have to be deconjugated by gut microflora before absorption can take place (Bingham *et al.*, 1998). Structurally related flavonol glucosides are absorbed by an active transport system (Hollman *et al*, 1995). However, it is unclear if isoflavone



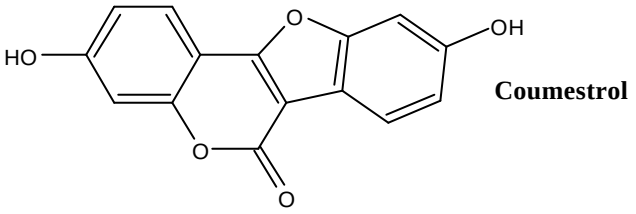
glucosides are absorbed as no studies have looked at the absorption of conjugated versus unconjugated isoflavones.

Figure 3: Metabolism of sissotrin and formation of equol *via* formononetin and daidzein.

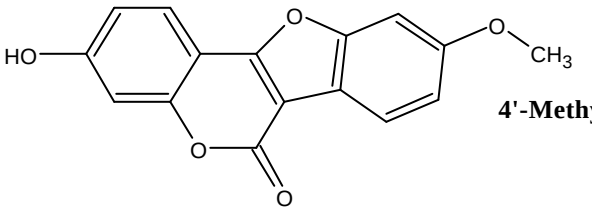
13. Following absorption of the aglucones, the isoflavones are reconjugated with sulphate and glucuronide and excreted (Bingham *et al.*, 1998). Gut microflora can also modify estrogenic isoflavones into more active forms. The methylated isoflavones, formononetin and biochanin A are demethylated by gut microflora to daidzein and genistein, respectively. Daidzein can then be converted into the more potent estrogen, equol (Bingham *et al.*, 1998) (see figure 3). The absorption, distribution, metabolism and excretion of phytoestrogens will be discussed in greater detail in a subsequent paper.

Coumestans

14. The coumestans, coumestrol and 4'-methylcoumestrol, are structurally and biosynthetically related to the isoflavones (see figure 4) (Humfrey, 1998). They are found predominantly in clover and alfalfa plants (Miksicek, 1993) and to a lesser extent in soybean sprouts (Reinli & Block, 1996) and so are rarer components of the human diet (Aldercreutz 1997).



Coumestrol



4'-Methylcoumestrol

Figure 4: The coumestans: coumestrol and 4'-methylcoumestrol.

Lignans

15. The lignans comprise a class of phytoestrogen compound that, like the isoflavonoids, also contain a diphenolic ring system but based around a 2,3-substituted dibenzylbutane skeleton (Mazur & Aldercreutz, 1998). In contrast to the major isoflavone aglucones, they are stereoisomeric and are found in Nature as racemic mixtures (Setchell & Adlercreutz, 1988).

16. Foodstuffs such as grains, seeds, in particular linseed, as well as other rich fibre foods have the highest concentrations of these compounds (Morton, 1997). **Secoisolariciresinol**, **matairesinol**, **lariciresinol** and **isolariciresinol** are the principal lignans found in plants (Setchell & Adlercreutz, 1988). They can occur in foods as aglucones or as mono and diglycosides (Aldercreutz *et al.*, 1995a; Aldercreutz *et al.*, 1995b; Tou *et al.*, 1998) (see figure 5). Little is known about the estrogenic potency of these compounds, but they are thought to be converted by gut microflora in a series of reactions to the weakly estrogenic, **enterolactone** and **enterodiol** (Setchell & Adlercreutz, 1988).

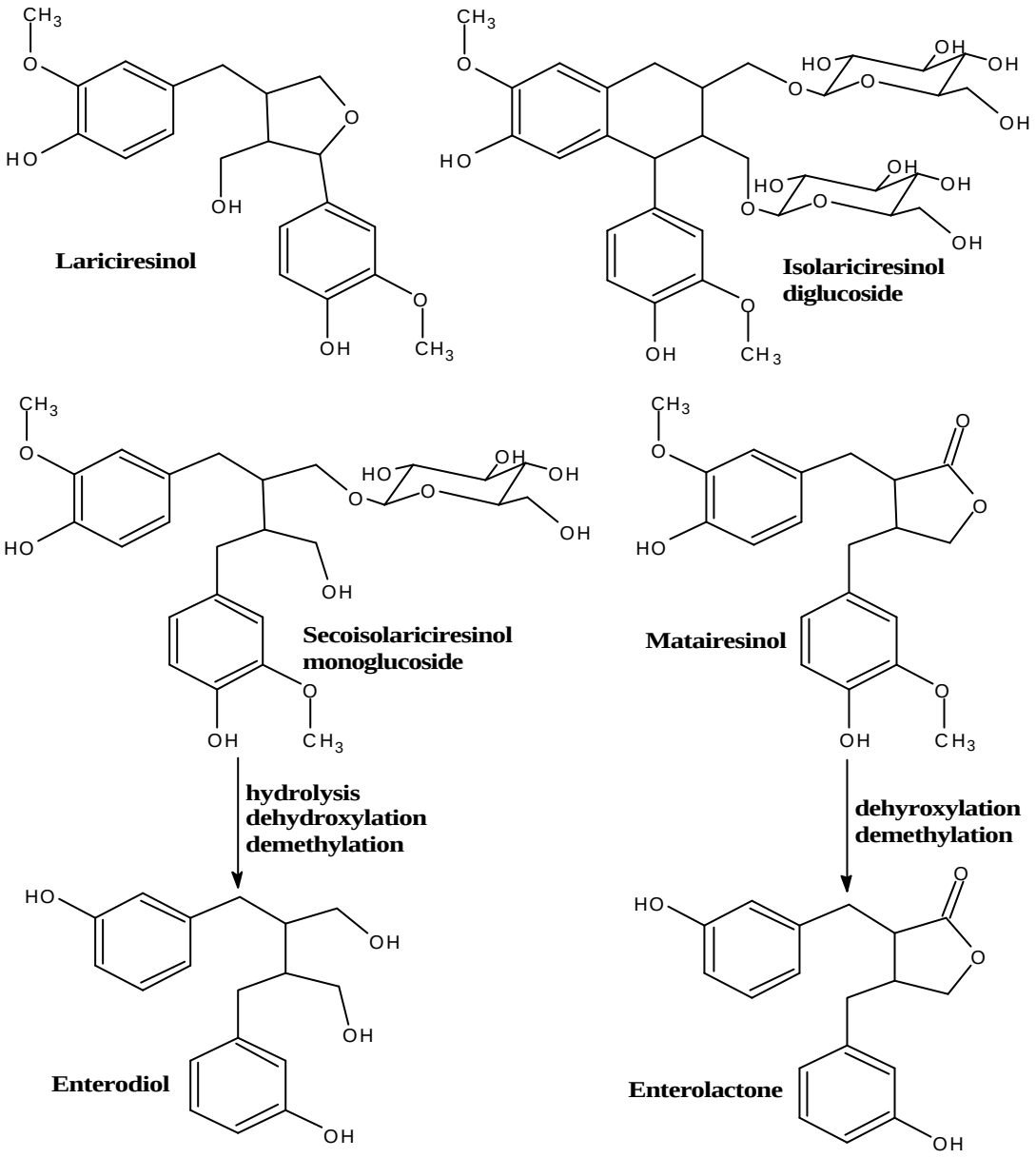


Figure 5: The lignans: lariciresinol, isolariciresinol diglycoside, secoisolariciresinol monoglycoside, matairesinol, enterodiol and enterolactone.

Prenylated flavonoids

17. A further class of flavonoids (Milligan *et al.*, 1999) have recently been identified from hops. These flavonoids are substituted with prenyl groups (Kitaoka *et al.*, 1998; Miyamoto *et al.*, 1998) and to date, **8-prenylnaringenin** (see figure 6) is the most potent, intermediate between coumestrol and genistein, in terms of estrogenicity (Kitaoka *et al.*, 1998).

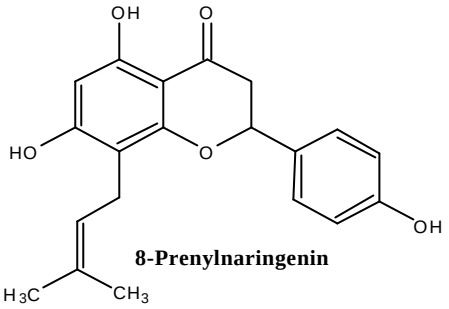
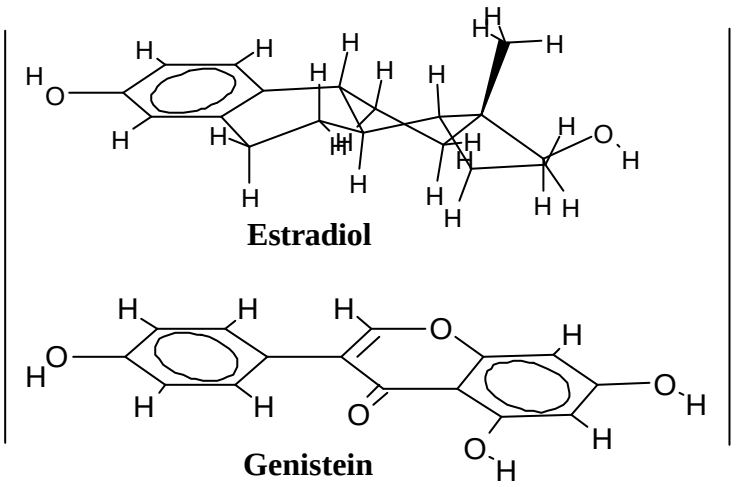


Figure 6: 8-prenylnaringenin.

Biological activity

18. Phytoestrogens share structural similarities with the female hormone, 17 -estradiol and this forms the basis of their estrogenic activity (see figure 7). The relative potencies of phytoestrogens with respect to 17 -estradiol varies depending on the assay used however, inter-assay orders of estrogenic potency are similar with the following order, 17 -estradiol >> coumestrol > 8-pentenylnaringenin > genistein > equol > daidzein > biochanin A =



formononetin. Estrogenic potencies of the lignans relative to 17 -estradiol have not been reported. Phytoestrogen potencies will be discussed in greater detail in a subsequent paper.

Figure 7: The structural similarity of the female hormone, estradiol and the isoflavone, genistein.

Review of Analytical Methodology

Introduction

19. The diverse structures and chemical properties of phytoestrogens and their metabolites, in addition to the range of matrices of which they are constituents, makes phytoestrogen isolation and analysis particularly challenging. Initially, phytoestrogens were analysed using imprecise and insensitive techniques such as thin-layer and paper chromatography. However, with the development of increasingly sensitive and accurate analytical technologies the ability of analysts to isolate, detect and quantify phytoestrogens has advanced considerably. As a result, the information available on phytoestrogen levels in foodstuffs and biological matrices has increased significantly in the last 5 years.

Isolation of phytoestrogens

20. Phytoestrogens have been isolated from a range of solid and liquid foods as well as biological matrices such as plasma/serum, urine and faeces. As minor components of complex mixtures, phytoestrogens must first be separated from the bulk of the matrix constituents and purified prior to analysis. The complexity of the matrix, the analytical method of detection and the analyte determine what extraction and purification steps are required.

Extraction methods for phytoestrogens

21. Due to the differing physical and chemical properties of phytoestrogen analytes extraction from food and biological matrices is often problematic and laborious. Losses that occur, due to inefficient extraction, should be accounted for. A number of analytical laboratories have established their own protocols to isolate, purify and analyse phytoestrogens from food and biological matrices (example given in figure 8). Extraction methods either involve organic solvent/aqueous buffer (Setchell *et al.*, 1997) or solid-liquid (Setchell & Adlercreutz, 1988) systems to remove analytes from matrices.

Extraction efficiency & the use of standards

22. Errors in extraction can be accounted for by the use of standards (Adlercreutz *et al.*, 1992). **Reference standards** are pure well-characterised samples that are used to calibrate or assess a measurement method or assign values to analytes (Thompson & Wood, 1993). **Internal standards** are compounds added to the sample or sample matrix to assess a method or assign values to analytes.

23. Prior to extraction, known amounts of internal standards, with similar physical and chemical properties to the analytes are added to the sample (Franke *et al.*, 1994; Mazur *et al.*, 1996). Alternatively standards of the analytes themselves are added to a duplicate sample of the matrix. The concentration of standard extracted is then used to quantify the extraction efficiency and correct for any losses (Franke *et al.*, 1994; Mazur *et al.*, 1996; Liggins *et al.*, 1998a, 1998b). The use of internal standards is preferable to the parallel analysis using standards as the correction is based on analysis of the sample itself.

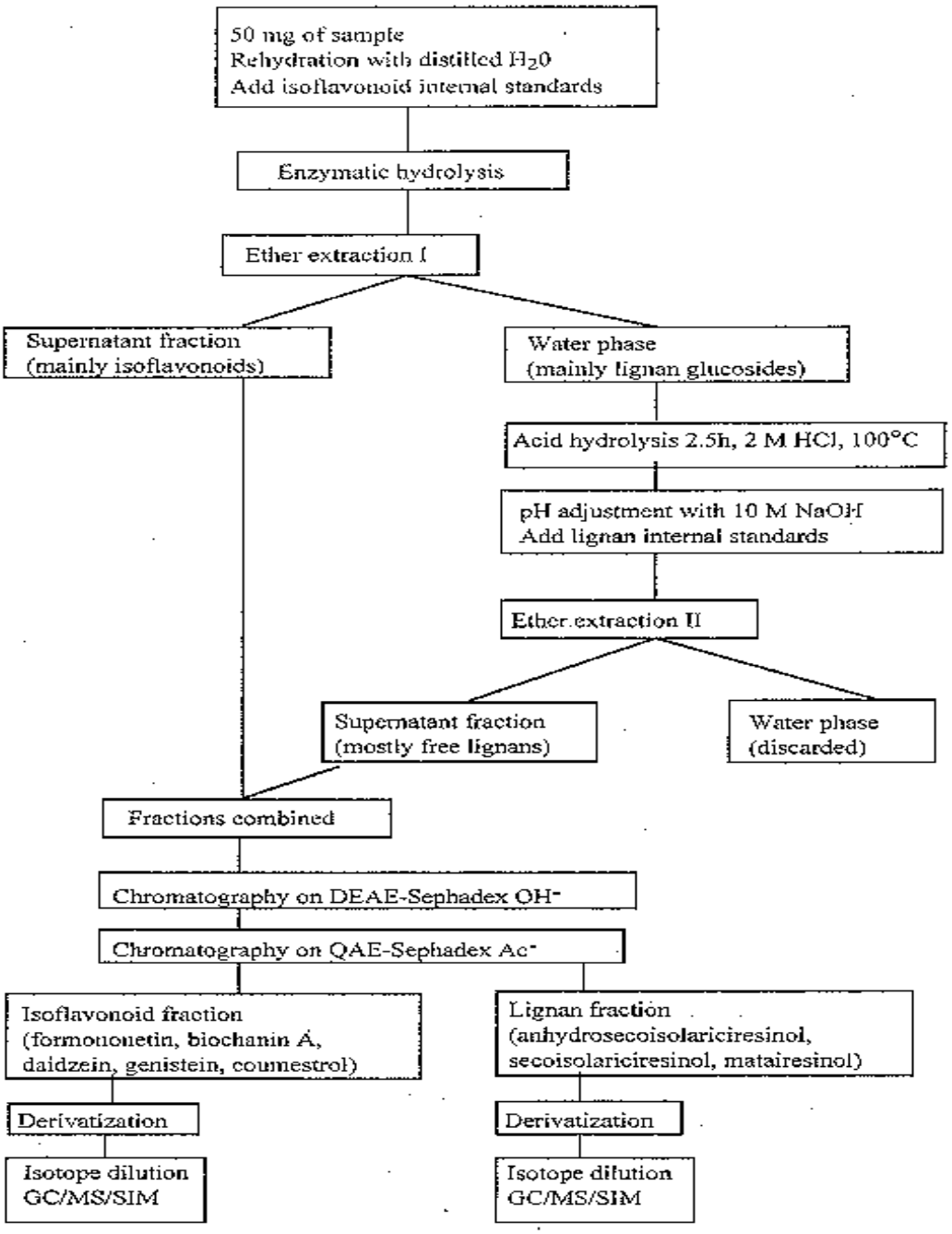


Figure 8: Flow diagram of an analytical procedure to measure isoflavonoids and lignans (taken from Mazur & Adlercreutz, 1998).

Analytical methods

24. The techniques most widely used for the analysis of phytoestrogens from biological matrices are:
(a) Reversed phase high performance liquid chromatography with ultra-violet detection (HPLC-UV).
(b) Gas chromatography with mass spectrometric detection (GC-MS).
(c) Liquid chromatography with mass spectrometric detection (LC-MS).

HPLC-UV

25. HPLC-UV is a relatively rapid method for phytoestrogen analysis. Phytoestrogens, once isolated from the matrix, can be directly resolved by HPLC and quantified by UV spectrometry. This allows simultaneous purification and quantification of complex mixtures of phytoestrogens. Quantification is achieved by creating calibration curves with reference standards (Xu *et al.*, 1995; Fukutake *et al.*, 1996; Murphy *et al.*, 1997). The method can incur overestimates of analyte concentrations as the UV absorbance of the analyte can be affected by co-eluting constituents of the sample, that are absent from reference solutions.

HPLC-UV: analytical limitations

26. HPLC-UV has been applied to the analysis of phytoestrogens in foodstuffs (Reinli & Block, 1996). The limits of detection for HPLC-UV methods are generally in the range of >1-2 ng/mL. Thus, UV detection is sensitive enough to measure phytoestrogen concentrations in foods and plants rich in phytoestrogens (Setchell & Adlercreutz, 1988; Mazur and Adlercreutz, 1998) but it is not often used to analyse foods with low levels of phytoestrogens (Mazur *et al.*, 1998) and biological samples (Adlercreutz *et al.*, 1993).

27. In the absence of standards of the other analytes measurements of isoflavone glucosides have used calibration curves from reference standards of the aglucones. This assumes that the UV absorbances of conjugated analytes do not differ to those of the glucone reference standards (King & Bursill, 1998). This assumption that the UV properties of the glucosides are identical to the aglucones is often not experimentally tested. UV extinction coefficients for genistein and genistin published by Coward *et al.* (1993)

suggest that the UV extinction coefficients differ with values for the glucoside and aglucone of 41.7 x 10^3 and 37.3 x 10^3, respectively. When the relevant reference materials are available such UV differences can be derived and results recalculated.

GC-MS

28. GC-MS is widely used in phytoestrogen analysis and is sufficiently sensitive to measure levels of phytoestrogens in the <ng/mL range (Setchell *et al*., 1987). Therefore, GC-MS is often used to measure the concentrations of phytoestrogens present in biological samples such as plasma and urine, as well as foods (Adlercreutz *et al.*, 1993; Mazur *et al.*, 1996; Mazur *et al.*, 1998). GC resolves the different components of compound mixtures.

29. Quantification of the analytes is usually done with the use of internal standards (Setchell & Adlercreutz, 1988; Setchell *et al*, 1997). The analytes of unknown concentration are measured with respect to the signal produced by the internal standard. The use of internal standards in this way is advantageous as both the internal standard and the analyte are measured under exactly the same conditions.

GC-MS: analytical limitations

30. Phytoestrogens must be chemically treated prior to analysis (Setchell & Adlercreutz, 1988). GC temperatures degrade glycosides on the GC column, reducing compound resolution. Chemical or enzymatic hydrolysis is used to remove conjugated groups and release the aglycones (Mazur *et al.*, 1996; Liggins *et al.*, 1998a). Enzymatic methods of hydrolysis are preferable for isoflavones as they can be unstable under acid hydrolysis conditions (Liggins *et al.*, 1998a). Lignan glucosides are resistant to enzymatic hydrolysis and require strong acid to release the aglucones (Mazur *et al.*, 1996). Once the phytoestrogens are hydrolysed, the aglycones are derivatised with a silylating agent to prevent the analytes binding irreversibly to the GC column. This treatment prerequisite for GC-MS analysis means that it is not possible to analyse the chemical forms of the analytes as present in the sample matrix. As a result analytical results are expressed as “total phytoestrogen”.

LC-MS

31. LC-MS is more sensitive than HPLC-UV and can measure <ng/mL concentrations of phytoestrogens (Cimino *et al.*, 1999). In addition, LC-MS, in contrast to GC-MS, does not require hydrolysis and derivatisation of the analytes prior to analysis. Therefore, the chemical integrity of the sample is preserved and information on the chemical identity of the isoflavone in the matrix can be obtained and need not be converted into a value of "total phytoestrogen".

LC-MS: analytical limitations

32. The use of LC-MS is dependent on the availability of suitable internal standards and this limits the compounds that can be analysed. The ionisation of different compounds varies considerably in a mass spectrometer and therefore, labelled internal standards of all the analytes are required. Many of these labelled standrds are not available. For this reason laboratories analysing isoflavones by LC-MS have hydrolysed samples prior to analysis and used the aglucone materials available (Cimino *et al.*, 1999). Thus, the advantage of non-destructive sample preparation that LC-MS offers over GC-MS has been precluded by lack of suitable standards.

Limitations of analysis: importance of standard materials

33. The analysis of many phytoestrogens is limited by the unavailability of appropriate internal standards and reference materials for phytoestrogen glucosides and phytoestrogen derived metabolic products. This has restricted the analysis of many compounds by the techniques described (Liggins *et al.*, 1998a).

34. All of the analytical methods described are dependent on the use of standards for accurate quantification. Inaccuracies can be introduced at any stage in the analysis from:
(a) incomplete extraction of analytes.
(b) inefficient hydrolysis of conjugates.
(c) errors in analyte measurements.

Reference & internal standards

35. Such errors can be accounted for during each procedure by the judicious use of standards (Adlercreutz *et al.*, 1992). **Reference standards** are pure well-characterised samples that are used to calibrate or assess a measurement method or assign values to analytes (Thompson & Wood, 1993). **Internal standards** are compounds added to the sample or sample matrix to assess a method or assign values to analytes. In MS-based methods isotopically labelled materials of the analytes can be used. Ideally these should differ by several mass units to allow differentiation of the two species in the mass spectrometer.

36. The lack of suitable standard materials has meant that many early studies of phytoestrogen levels in foods and biological matrices have not used reference or internal standards and assumed that extraction and/or hydrolysis processes are completely efficient (Adlercreutz *et al.*, 1995b) leading to underestimation of the reported results.

37. A number of studies have tried to circumvent this problem by using compounds that are judged to have sufficiently similar physicochemical properties to those of the analyte, such as flavone, as standards (Adlercreutz *et al.*, 1982; Setchell *et al.*, 1997; Heinonen *et al*, 1999). This approach is an improvement as a estimate of extraction and/or hydrolysis efficiencies can be made however, errors of over- or under-valuation of results could still be introduced.

Preparation of reference materials

38. Standard reference materials can be isolated and purified from natural sources (Farmakalidis & Murphy, 1985) or chemically synthesised (Murphy *et al.*, 1997). Synthetic routes to reference compounds are advantageous as pure fully characterised compounds can be supplied in large quantities.

Preparation of internal standards

39. Synthetic routes can be used to incorporate isotopic labelled standards of the analytes. Isotopic labelling is of great benefit as labelled compounds have identical physical and chemical properties to those of the analyte.

Radiolabelled compounds useful as tracers in metabolism studies can also be synthesised.

^{2}Hydrogen labelled standards

40. Internal standards of genistein, daidzein and equol incorporating deuterium (^{2}H) labels have been synthesised (Axelson *et al.*, 1982; Adlercreutz *et al.*, 1991; Morton *et al.*, 1997). Some lignans incorporating ^{2}H-labels have also been synthesised (Mazur *et al.*, 1996).

41. Deuterium labelling can be achieved by hydrogen-deuterium exchange reactions and therefore, does not require extensive synthetic chemistry. However, ^{2}H-labels are unstable and can exchange with hydrogen, particularly under acidic conditions used in conjugate hydrolysis. This leads to loss of the label during sample manipulation or analysis resulting in inaccurate evaluation of extraction/hydrolysis efficiency and/or analyte measurement (Adlercreutz *et al.*, 1993).

^{13}Carbon labelled standards

42. The Food Standards Agency Phytoestrogen R & D programme has funded a project to synthesise isoflavones and lignans incorporating chemically stable carbon (^{13}C) labels (Synthesis of labelled and unlabelled isoflavonoid phytoestrogens, Dr N. Botting, University of St. Andrews). To date such labels have successfully been introduced into the isoflavone aglucones, genistein, daidzein and formononetin both as single (Whalley *et al.*, 1998) and triple (Fryatt *et al.*, 1999) ^{13}C-labels. Labelled standards of the glucones, metabolic products and lignans are currently under preparation. These materials have been supplied to the laboratories undertaking phytoestrogen analysis in Food Standards Agency programme.

Quality assurance for phytoestrogen analysis

43. Analytical laboratories run internal quality control procedures to check their analytical protocols (Adlercreutz *et al.*, 1991; Lampe *et al.*, 1994; Hutchins *et al.*, 1995; Murphy *et al.*, 1997; Song *et al.*, 1998). This can involve multiple analysis of the same sample, analysing standard materials

along with batches of the analytical samples and monitoring the results of repeat analysis of test materials.

44. Quality assurance (QA) schemes allow inter laboratory comparisons of reported results. This usually involves analysis of defined materials by participating laboratories supplied by a co-ordinating laboratory. QA schemes provide further confidence in the accuracy, precision and procedures of laboratories participating in a scheme.

45. The Food Standards Agency Phytoestrogens R & D Programme has initiated a pilot QA scheme in which the programme's analytical laboratories participate. co-ordinated by Central Science Laboratories, York. The results from participating laboratories were found to be in satisfactory agreement.

Summary

46. The state-of-the-art of phytoestrogen analysis is constantly evolving and improving as interest in these compounds has grown. As a consequence it may be considered that those studies conducted more recently have implemented procedures that could produce results of greater accuracy.

Secretariat March 2000

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