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Natural and anthropogenic environmental oestrogens: the scientific basis for risk assessment*

Naturally occurring oestrogens in food

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Abstract: Edible plants contain a number of natural compounds which mimic the biological effects of oestrogens by virtue of their ability to bind to and activate the nuclear oestrogen receptors. These hormone-like diphenolic phyto-oestrogens of dietary origin include isoflavonoids, coumestans and lignans. Our interest in these phyto-oestrogens derives from the results of epidemiological studies on diet and Western diseases including hormone-dependent cancers as well as coronary heart disease. Incidences of the diseases in question are lower in peoples of Asia compared to inhabitants of industrialized American and European countries. Using isotope dilution gas chromatography-mass spectrometry method we have identified and measured in foods the precursors of the biologically active compounds detected in plasma of subjects living in areas with low cancer incidence. Biochanin A, formononetin, daidzein, genistein, and coumestrol, and the lignans matairesinol and secoisolariciresinol have been found to possess oestrogenic, anti-oestrogenic, antioxidative, antiviral, antibacterial, insecticidal or fungistatic properties and they have been shown to be antiproliferative in relation to many types of tumours in cell culture. We report quantitative results for these plant oestrogens measured in soybeans and other legumes, oilseeds and nuts, grain and cereals, berries and fruits, cruciferous, allium and other vegetables, and beverages such as tea and coffee.

INTRODUCTION

Plant foods, in addition to their traditional nutritional values, contain certain non-nutritional phytochemicals that may exert long-term health-promoting affects in the human. Important pharmacological effects of plants in the human have been acknowledged and used in medicine for thousands of years. However, until now mechanisms by which plant-derived compounds act in human have only partially been elucidated. Two important pathways for the biological actions of plant-derived chemicals have been suggested—these include binding either to hormone receptors or to enzymes that metabolize hormones.

On the other hand, epidemiology has established connection between semivegetarian diet in some Asian countries and a reduced incidence of many chronic and degenerative diseases (i.e. the major hormone-dependant cancers, colon cancer, atherosclerosis and coronary heart disease), indicating that some unknown substances in this diet may contribute to homeostasis and thus play a role in the maintenance of health. These findings triggered research to identify nutritional biomarkers that could be responsible for the international variations in the incidence of these chronic diseases.

Given that all these diseases to various extent are associated with sex hormones or sex hormone metabolism, it was postulated that the Western diet, compared with the vegetarian or semivegetarian diet in some developing and Asian countries, may alter hormone production, metabolism or action at the

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cellular level (1–5) by some biochemical mechanisms. Since the sex steroid hormones are intimately involved in the signalling pathways for cell division and interact with growth factors, it is implied that agents that act on this system are likely to be involved in the carcinogenic and atherosclerotic processes.

The detection and identification in human body fluids of two groups of active principles, lignans (Fig. 1) and isoflavonoids (Fig. 2), both of plant origin with molecular weights and structures similar to those of steroids, suggested that they could be important modulators of the human hormonal system and hormone action (2–3, 6–8). The plant lignan and isoflavonoid glycosides are transformed by intestinal bacteria to hormone-like compounds (9–12). The mechanism(s), through which the phyto-oestrogens may influence sex hormone production, metabolism and biological activity and exert anticancer, cancer-protective, antiatherogenic, cardioprotective, bone-maintaining effects, seems to depend, at least in part, on their mixed oestrogen agonist-antagonist properties. Furthermore, these weakly oestrogenic molecules were demonstrated to effect intracellular enzymes, protein synthesis, growth factor action, malignant cell proliferation, cell differentiation, cell adhesion, angiogenesis, and apoptosis. Strong experimental evidence (5, 13) suggests that both lignans and isoflavonoids are among the dietary factors affording protection against cancer and atherosclerosis.

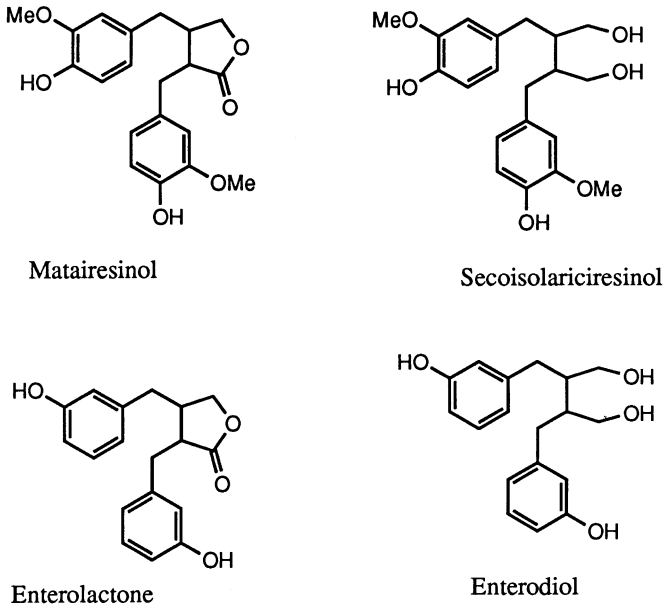

Fig. 1. Structures of plant and mammalian lignans.

Despite of immense laboratory research efforts, *in vitro* and *in vivo* animal studies, which reveal interesting properties of phyto-oestrogens and food containing these compounds as inhibitors of carcinogenic and atherosclerotic processes, some basic questions are not yet answered. The mechanisms hypothesized above as influencing cancero- and atherogenicity have not yet been examined directly in epidemiological studies at the substance level. Considering the chemoprotective role of these non-nutrients it is of importance to know more about their presence in terms of quantity and quality in the human diet. Despite the wealth of studies on food groups such as fruits and vegetables and chronic diseases (i.e. cancer and coronary heart disease), there is a dearth of studies on active ingredients (i.e. phyto-oestrogens) in foods because of the lack of reliable food compositional data on these substances. Lignans and isoflavonoids are not listed along with nutrients in tables of food composition neither it is possible to calculate the intake. Consequently, we have developed a method that allows the identification of foods that contain phyto-oestrogens and enables the assessment of the intake of all biologically important isoflavonoids and lignans, and facilitates research to clarify the role dietary phyto-oestrogens may play in various aspects of health and disease. The results of our analyses have been published in a few separate communications cited below. Herein we present our recent data on naturally occurring phyto-oestrogens in diet.

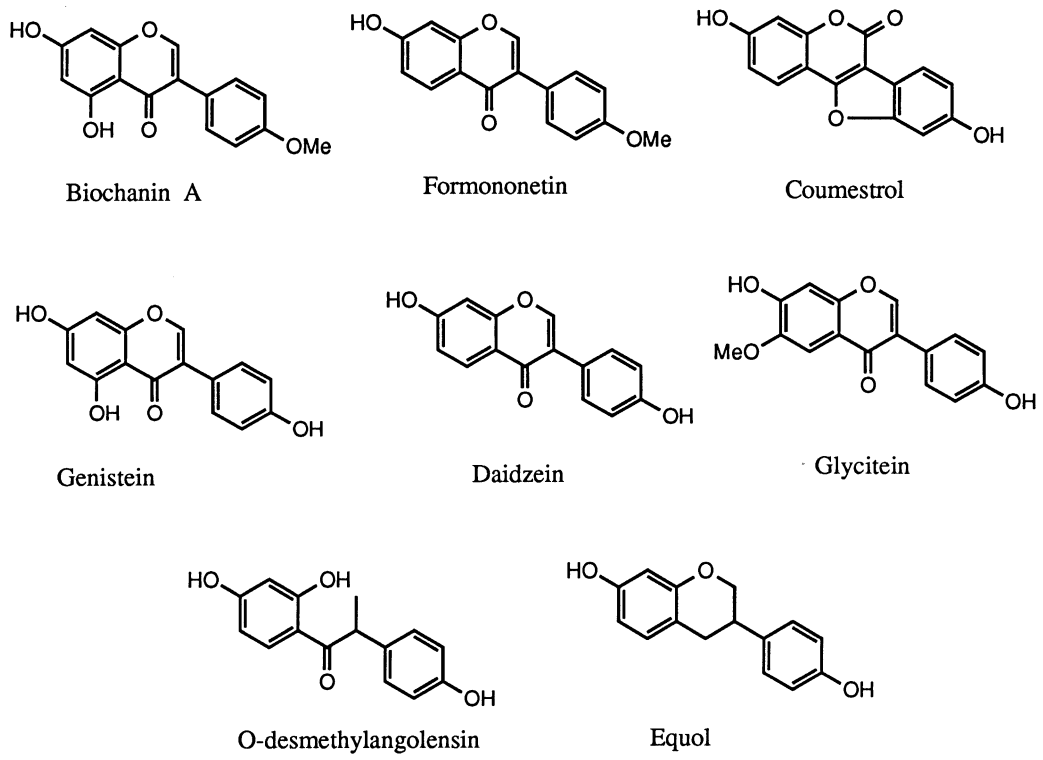

Fig. 2. Structures of isoflavonoids and coumestrol.

PLANT OESTROGENS—ORIGIN AND EVOLUTION

Plant foods contain at least twelve thousand natural chemicals produced for structural, hormonal, attractant and chemoprotective purposes. A remarkable diversity has been described for naturally occurring phyto-oestrogens, which share with steroidal oestrogens an ability to activate the oestrogen receptor and which have been shown to exert oestrogenic effects on the genital tract of female animals. Consequently, a large diversity of action has been attributed to naturally occurring weakly oestrogenic compounds in the large flavonoid family of plant secondary metabolites as well as to plant lignans. The flavonoids occur in several structurally and biosynthetically related classes such as flavones, flavonols (3-hydroxyflavones), anthocyanins, flavanones, isoflavonoids (isoflavones, coumestans), and chalcones. The number of non-steroidal compounds with oestrogenic activity in edible plants is constantly growing. The main known phyto-oestrogens are the isoflavones daidzein, genistein, formononetin, and biochanin A and the coumestan coumestrol. The lignans secoisolariciresinol (SECO) and matairesinol (MAT), precursors of the hormone-like mammalian lignans, are of particular interest due to their abundance in plants.

The biosynthesis pathways of flavonoid and steroid precursors presumably have crossed more than once on the long evolution route from early procaryotic organisms billion years ago to angiosperms 100 million years ago. The basic steroid skeleton has remained unchanged during about 1.5–2 billion years, and the complicated hormonal system in multilocular organisms has developed much later. Steroids are products of bacterial, fungal and plant metabolism while, according to present knowledge, flavonoids are produced only by fungi and plants, whereas lignans are exclusively plant-derived chemicals.

The evolution of the flavonoids has to be understood in the light of their function in the environment in which they occur (14). The most important activities of flavonoids are dependent on the organisms in which they are present and is also related to flavonoid structure. As typical phenolic compounds the flavonoids act as potent antioxidants. As conjugated aromatic compounds they can act both as potent

screens against destructive UV light and as attenuators of physiologically active visible light. But the most remarkable properties with regard to interferences with viral, bacterial, fungal and animal reproduction, growth and development and feeding are revealed by few representatives of the flavonoids. These are especially isoflavones which, due to their structure, simulate steroidal and other controllers of growth and development in their potential predators, and lignans which in their polymeric form can bind proteins, including enzymes, and other polymers such as polysaccharides and nucleic acids. These chemicals being heterocyclic phenols exhibit a close similarity in structure to oestrogenic steroids. For example, the distance between the two aromatic hydroxyl groups in the nucleus of the isoflavones is almost identical to the distance between the C3 and C17 hydroxy groups of oestradiol, while the presence of a phenolic hydroxyl, a pre-requisite for the oestrogenic activity (15), accounts for the biological activity of these compounds.

The flavonoids, in a number about four thousand, have closely related structures, based on C15 heterocyclic nucleus of flavone and varying chiefly in the number of phenolic, methoxyl and other substituents. They are derived biosynthetically from the union of aromatic (hydroxycinnamyl coenzyme A ester) and aliphatic (malonyl coenzyme A) precursors. About 500 million years ago, on the evolution route of biosynthesis to the flavonoids the flavone apigenin, the basic flavonoid found along with sterols in the advanced blue-green algae (cyanobacteria), was a product of dehydrogenation of the C2–C3 bond in flavanones. The formation of flavones (16) in the blue-green algae, which inhabit the shores of lakes and streams, may have been a result of some mutation of systems which introduced double bonds into the rings of steroids. When about 420 million years ago plants invaded lands they began to stiffen the internal and outer cell of their stems for upright growth and for further protection of the cell walls against potential enemies. During the course of lignification, which involves polymerization of cinnamyl alcohols, lignans were formed. About 300 million years later, in the middle Cretaceous, the angiosperms arose and brought the essential changes, such as exploitation of compounds for colour, pigmentation and protection, in flavonoid evolution (16). At this stage the isoflavones and their congeners formed a main class of flavonoid compounds affording antifungal, antibacterial and antiviral protection. These compounds make up the bulk of the phenolic antibiotic phytoalexins, compounds which are synthesized only when infection has started. Some are also potent insecticides and can act as oestrogen mimics in mammals. Flavonoids secreted by plants may act as signals to initiate a co-operative activity: symbiosis between soil bacteria belonging to the Rhizobium family and legumes (17) that leads to formation of nitrogen-fixing nodules in the legume root. Isoflavonoids can be isolated from most plant tissues (18), including leaves, stems, roots, flowers, seeds and germs. In germs and sprouts these compounds occur in abundance (13) and seem to regulate physiological processes important for plant growth.

ISOFLAVONOIDS AND LIGNANS—CHEMISTRY AND METABOLISM

Isoflavonoids are a large and very distinctive subclass of the flavonoids. These compounds differ structurally from other classes of the flavonoids in having the phenyl ring (B-ring) attached at the 3- rather than at 2-position of the heterocyclic ring. In addition, the isoflavonoids differ by their greater structural variation and the greater frequency of isoprenoid substitution. Isoflavones are isomeric with the more widely occurring flavones, and genistein is derived biosynthetically by an aryl migration from the same chalcone precursor as that which gives rise to the flavone apigenin. Isoflavonoids are more restricted in plant kingdom than other flavonoids, since they are found regularly in only one subfamily of the Leguminosae, the Papilionoideae. They have been recorded occasionally in a few other families, such as the Compositae, Iridaceae, Myristicaceae and Rosaceae. The knowledge of their natural occurrence is, at least in the light of our research, imperfect. This is, however, mostly due to the lack of sufficiently sensitive methods for screening plant tissues and plant-derived foods for the presence of isoflavonoids.

Isoflavones constitute the largest group of natural isoflavonoids with about 364 aglycones reported. In this subclass the most investigated and interesting compounds with regard to oestrogenicity are genistein, daidzein, biochanin A and formononetin. Genistein (4′,5,7-trihydroxyisoflavone) is the most active principle with the highest binding affinity for the oestrogen receptor (19). The methoxy derivative, biochanin A, does not bind to the oestrogenic receptor but is oestrogenic *in vivo* (20–21). Daidzein (4′,7-dihydroxyisoflavone) has a higher binding affinity for the oestrogen receptor than its methoxy derivative,

formononetin, but both are weak oestrogens *in vivo* (19). Methylation could be the mechanism through which the oestrogenic potency of isoflavones is reduced (22). The differential potency between genistein and daidzein could instead be referred to the presence of the 5-hydroxyl group of genistein (22).

The following isoflavonoid phyto-oestrogens have been identified or detected in human urine: formononetin, methylequol, daidzein, dihydrodaidzein, *O*-desmethylangolensin, genistein, and 3′,7-dihydroxyisoflavan and some other metabolites (1, 6, 23–28). Daidzein, genistein, equol, and *O*-desmethylangolensin have been measured (29–32) by isotope dilution gas chromatography-mass spectrometry in the selected ion monitoring mode (ID-GC-MS-SIM) in human plasma, and faeces.

Studies on the origin, formation and metabolism of the phyto-oestrogens in animals (33) and in man (34–36) have been reviewed. Isoflavones and lignans show a similar pattern of metabolism and disposition in animals and humans, while the metabolism of coumestans has not been characterized. When consumed, the plant isoflavonoids and lignans undergo metabolic conversions in the gut resulting in the formation of hormone-like compounds with the ability to bind with low affinity to oestrogen receptors and with weak oestrogen activity (33, 36).

The metabolism of formononetin, daidzein, biochanin A, and genistein has been studied particularly in sheep (37–38). Biochanin A is converted to genistein, which is further metabolized to p-ethylphenol and dihydrogenistein. Formononetin is converted to daidzein and daidzein to *O*-desmethylangolensin, equol and some other metabolites. The metabolism varies in animals and metabolism in man may not be identical with that one found in sheep or cattle (39). The hydrolysis of the flavonoid glycosides takes place in the proximal colon and several bacteria have been found which produce the necessary enzyme (40–41). Recent studies indicate that also the lignan glycosides are hydrolysed in the proximal colon (42, and unpublished observation).

In man, equol (8, 43) and *O*-desmethylangolensin are most likely formed, as in sheep, by intestinal bacterial action from formononetin and daidzein present in foods such as soy products (36). Some people are unable to produce equol or they excrete this isoflavone in very low amounts (8, 36, 43). Equol excretion also depends on diet; a high-fat and -meat diet in Japanese subjects is associated with higher equol values (44).

Because equol and other isoflavone metabolites occur in cow milk (45), their presence in human urine is not necessarily the consequence of intestinal metabolism of their precursors. In countries, like Finland, with low soy consumption the low basal levels of isoflavonoids found are therefore probably a result of intake of dairy products and meat. Even fish, given soy-containing foods, including some ready formed isoflavonoid metabolites, may be responsible for the small amounts that are excreted in urine of Finns, or they may result from food (e.g. bread) additives containing minor amounts of soy protein with isoflavonoids (unpublished observations).

As oestrogens (46–47) and flavonoids (48), the isoflavonoids seem to undergo an enterohepatic circulation, at least in the rat (10, 49–50). After absorption in the small intestine, isoflavones and lignans are conjugated with glucuronic acid and sulfate by hepatic phase II enzymes (UDP-glucuronylosyltransferases and sulfotransferases). Lignan and isoflavone conjugate profiles in human urine suggest that glucuronic acid is the primary moiety (51). Like endogenous oestrogens, these conjugates are excreted both in urine and in bile and undergo enterohepatic circulation. After excretion into bile, conjugated isoflavones and lignans can be deconjugated once again by gut bacteria. However, there are no data on isoflavonoid metabolites in human bile. Deconjugation may promote reabsorption, further metabolism, and degradation in the lower intestine (36, 52).

Coumestrol (3,9-dihydroxy-6H-benzofuro[3,2-c][1]benzopyran-6-one), the most potent of coumestans, has higher binding affinity for the oestrogen receptor than genistein (53). This is consistent with the receptor binding model that appears to depend upon a phenolic group in the 4′ position of isoflavones and the 12′ position of coumestans.

Lignans, by definition, are compounds possessing a 2,3-dibenzylbutane structure, and such compounds are known to exist as minor constituents of many plants where they form the building blocks for the formation of lignin in the plant cell wall. Nearly two decades ago two cyclically occurring unknown compounds, now known as enterolactone and enterodiol, were detected in the urine of the

female vervet monkey, and women (54–56), and subsequently identified separately and independently by two groups (55, 57). Furthermore, small amounts of four plant lignans—MAT (immediate precursor of enterolactone), lariciresinol, isolariciresinol and SECO (immediate precursor of enterodiol)—were identified along with some other metabolites (23, 58). Enterolactone, enterodiol, and later MAT and SECO were measured by ID-GC-MS-SIM in human plasma, urine and faeces (29–30, 59–60). Enterolactone, alone or together with enterodiol, has also been measured in cow milk (45), human breast cyst fluid, saliva and prostatic fluid (5, 61).

The two major mammalian lignans, enterodiol and enterolactone, are the products of colonic bacterial metabolism of the plant lignans SECO and MAT, respectively (9–12). The compounds occur mainly in the glycosidic form in the plants and these glycosides are hydrolyzed in the proximal colon. The colonic microflora convert MAT to enterolactone and SECO to enterodiol, and the latter is readily oxidized to enterolactone (11–12, 36). At least in rats the plant lignans undergo an enterohepatic circulation and it is most likely that this is the case in man also as shown for phenolic oestrogenic steroids (46–47). Lignans are excreted in both urine and faeces, and in human subjects the faecal excretion pathway seems to be more important than it is for oestrogens (47, 62), since the amounts are similar or only slightly lower in faeces than in urine (32). This could be explained by assuming that some of the formed mammalian lignans escape absorption.

Administration of antibiotics almost completely eliminates the formation of enterolactone and enterodiol from plant precursors in the gut (9, 63) and later leads, after initial rapid lowering of the lignan levels in urine, to a relative increase in the enterodiol/enterolactone ratio.

PHYTO-OESTROGENS: OESTROGENICITY VERSUS ANTI-OESTROGENICITY

The oestrogenic activity of clover, one of the richest source of phyto-oestrogens, was first described over 50 years ago following the observation that sheep feeding on pastures that containing clover demonstrated hyper-oestrogenization and infertility (64). Crucial in demonstrating that phyto-oestrogens shared a common mechanism of action were studies in experimental systems in which phyto-oestrogens competed with radiolabelled oestradiol for binding to the oestrogen receptor and elicited oestrogenic responses in oestrogen-responsive tissues and cells (33). However, a number of representatives of three main chemical classes of phyto-oestrogens (the isoflavonoids, coumestans and lignans) showed a different, generally low, oestrogenic potency in bioassays. Coumestrol, daidzein, genistein, equol, and *O*-DMA have been reported to bind the ER in cytosol preparations of sheep uteri with relative binding affinities of 5, 0.1, 0.9, 0.4, and 0.05% of oestradiol, respectively (19). Results of a recent *in vitro* study in human breast cancer cells (65) revealed that genistein had oestrogenic and ER-independent cell growth-inhibitory actions. Genistein, over a physiologically relevant concentration range, could serve both as a surrogate oestrogen agonist and as a growth regulator. On the contrary, definite anti-oestrogenic effects of phyto-oestrogens have been observed *in vivo*, because synthetic or natural oestrogens seem to be counteracted by administered isoflavonoids or their presence in the diet (66–67). Phyto-oestrogens, at concentrations of 100–1000 times higher than that of oestradiol (the achievable levels in human plasma after regular phyto-oestrogen consumption), have been considered to be able to compete effectively with endogenous mammalian oestrogens, bind to the ER, and prevent oestrogen-stimulated growth in mammals (68). On the other hand, equol, genistein and coumestrol have been found to act through oestrogen-receptor mediated processes and do not show any anti-oestrogenic effects in human breast cancer cells in culture (69–70). It is doubtful if any true receptor-mediated anti-oestrogenic effect of phyto-oestrogens at the cellular level exists, but other anti-oestrogenic mechanisms are possible.

Recent reports (71–72) identifying a novel rat oestrogen receptor β (ERβ) and a later study (73) on the ligand selectivities and the tissue distributions of both the ER subtypes ERα and ERβ have thrown new light on the oestrogenic activity of phyto-oestrogens. Both coumestrol and genistein exhibit a significantly higher affinity for ERβ protein than for ERα, which is interesting in the light of the high expression of ERβ mRNA in the secretory epithelial cells of the prostate, and the prostate cancer protective properties that have been associated with these compounds. Additionally, ERβ is expressed prominently in tissues such as the brain and urinary tract, and apparently also in breast cells (74).

No detectable oestrogenic activity of enterolactone and enterodiol was revealed in *in vivo* in mice (9) although these lignans bound weakly to rat uterine cytosol (J.H. Clark and H. Adlercreutz, unpublished 1986). *In vitro*, however, in four sensitive assays in tissue culture, including breast cancer cell lines, the lignans were stimulatory and the effect could be blocked by the anti-oestrogen Tamoxifen. No anti-oestrogenic properties were observed (75). In another study, enterolactone inhibited *in vivo* oestrogen-stimulated RNA synthesis in rat uterine tissue when administered 22 h before oestradiol (76). The concentrations of enterolactone were very low and it is doubtful whether this result can be repeated. We observed a stimulatory effect of enterolactone on MCF-7 breast cancer cells in the absence of oestradiol, but a slightly stimulatory or nonstimulatory concentration of oestradiol combined with a slightly stimulatory concentration of enterolactone did not cause stimulation or a tendency to inhibition (77). The enterolactone concentration was 1 µmol/L, which can be regarded as physiological. Enterolactone, but not enterodiol, was shown to stimulate pS2 expression in MCF-7 cells (70, 78). These diverging results are difficult to explain, but it has been suggested (32, 79) that the effect of exogenous weak oestrogens may be either agonistic or antagonistic depending on the level of endogenous oestrogens, and this has been experimentally confirmed with regard to coumestrol (79).

METHODS

Although many methods for the separation and quantitation of phyto-oestrogens in plant (food) extracts by high performance liquid chromatography (HPLC) have been described (80–84), their sensitivity and/or specificity is not sufficient for food samples with low concentrations of the compounds. Isoflavonoids and lignans were never measured in the same assay. In three studies (85–87) separate measurements of lignans in flaxseed have been performed. Most of authors report data for the richest sources, there is a little or no quantitative information on foods whose phyto-oestrogen content may be low but not zero and that may be commonly consumed in the population. Isoflavones and coumestrol content in 107 food items of British diet was explored (88) with HPLC and no detectable phyto-oestrogens were found in any of the samples. Isoflavonoid content of foods (particularly soy-derived) seem to depend on the sensitivity and specificity of the methodology. Particularly at low levels HPLC, being a blind detection system, may easily result in nonspecific results. Furthermore, natural variation occurs due to species differences, year of cultivation and region where the crop was produced.

Based on our experience with previously developed isotope dilution gas chromatography-mass spectrometry methods for the identification and quantitative determination of lignans and isoflavonoids in human urine (89), plasma (29–59) and faeces (32) an original methodology for the quantitative determination of the phyto-oestrogens formononetin, biochanin A, daidzein, genistein, and coumestrol and simultaneously the lignans SECO and MAT in plant-derived foods, has been developed. These compounds, after a three-step hydrolysis converting the diphenolic glycosides into their respective aglycones, are measured by ID-GC-MS-SIM using synthesized deuterated internal standards for the correction of losses during the procedure. The coefficient of variation of the method used was found to vary between 3.1 and 9.6% (concentration range 0.05 to 0.13 mg/kg) for the seven compounds. The mean recovery for all the analysed phyto-oestrogens was 99.5% and the sensitivity limit was approximately 0.02–0.03 mg/kg (standard deviation of the assays at low levels multiplied by 2–3). A flow diagram of the ID-GC-MS-SIM method used is shown in Fig. 3.

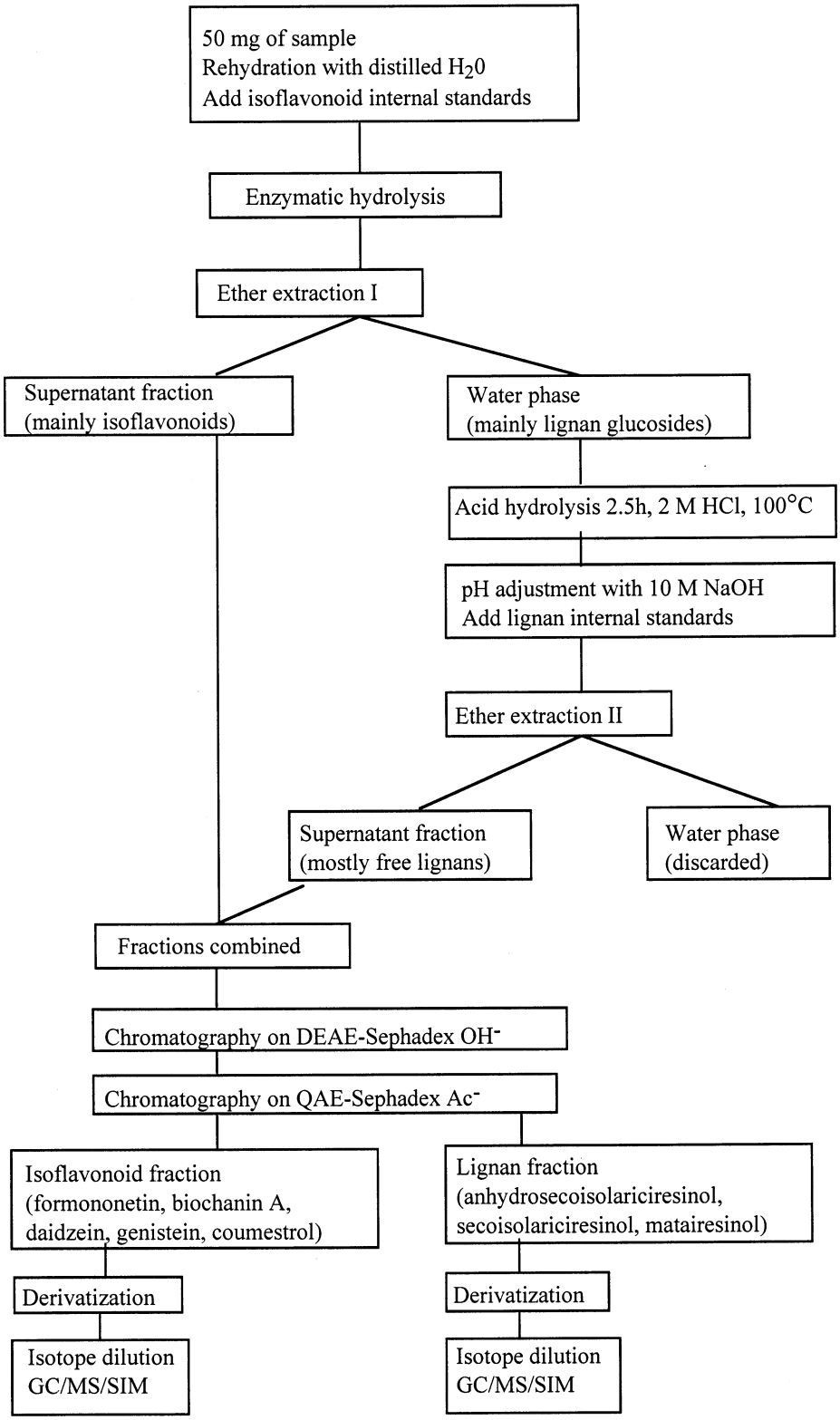

Fig. 3. Flow-diagram of the ID-GC-MS-SIM method for the determination of lignans and isoflavonoids in food samples.

PHYTO-OESTROGEN CONCENTRATIONS IN VARIOUS FOODS

The Leguminosae

The isoflavones enjoy a widespread distribution in most of the members of the Leguminosae family with its prominent high-content representatives such as soybean, clover, mung bean, alfalfa, peanut and kudzu (*Pueraria lobata*). As early as in the 1930s, rather high amounts (up to 100 000–300 000 µg/100 g) of the glycosides of the two isoflavones daidzein and genistein were detected and quantified in soybeans (90–92). Much later a third major compound, glycitein (4′,7-dihydroxy-6-metoxy-isoflavone), was found also mainly as a glycoside (glycitin) (93) (Fig. 2). Small amounts of these three compounds occur in the free form.

Soybeans and soy foods have been extensively investigated in regard to isoflavone content (82, 94–97). After normalization for differences in isoflavone molecular weights, these legumes and their products contain approximately 20 000–160 000 µg/100 g dry weight (dw) of isoflavones daidzein + genistein. These values in the literature agree well with ours.

Recently we have reported quantitative results for 48 cultivars of 16 common food legume species, and for 4 forage legume samples (all values given for dry weights) (98). The highest total concentration of isoflavones, with regard to edible seeds, was found in kudzu root (over 200 000 µg/100 g), followed by soybeans (ranged from 37 300 µg/100 g to 140 300 µg/100 g) and chickpea (1150–3600 µg/100 g). The same relation in concentrations held also for daidzein and its precursor formononetin. All soybeans analyzed proved to be the richest source of genistein, the most biologically active phyto-oestrogen. However, besides kudzu, significant amounts of this compound were measured in pigeon pea, groundnuts, pinto and haricot beans. The highest concentrations of its precursor, biochanin A, were found in *Cicer arietinum*, another commonly consumed legume species. All the legumes, except the 'Green split' pea, comprised daidzein and genistein, and, with a few exceptions, the amounts of genistein exceeded daidzein concentrations. Coumestrol was detected in most of the samples at very low concentrations (up to 10 µg/100 g); the soybean 'Santa rosa', kudzu leaf, and red clover contained more (105.0–1570 µg/100 g). The richest source of coumestrol in human food that we have found is mung bean sprouts which contain 20 times as much coumestrol (about 1000 µg/100 g) as do alfalfa sprouts (45 µg/100 g). In addition mung bean sprouts contain large amounts of daidzein (about 700 µg/100 g) and genistein (about 2000 µg/100 g). SECO at the concentration 1590 µg/100 g was detected in *Sophora japonica*. However, of the most commonly known and consumed legume species analyzed, the soybean and the peanut (*Arachis hypogaea*) contained the highest level of SECO (13.3–273 µg/100 g and 333.0 µg/100 g, respectively) but minor or no amounts of MAT could be detected. Generally, all the other legume items analyzed contained SECO (from 2.8 to 475.8 µg/100 g). MAT seems to be the rarest phyto-oestrogen in the Leguminosae plant family; although considerable amounts were found in black gram samples. Red clover, a perpetrator of 'clover disease' in sheep in Australia, was found to contain the highest concentrations of formononetin and biochanin A. These phyto-oestrogens were not found in alfalfa, another common forage legume, at all. The content of lignans in the forage legumes was lower and ranged from trace to 19.4 µg/100 g (0.5 µmol/kg).

Phyto-oestrogen content of selected legumes and soybean products is shown in Table 1.

Oilseeds and nuts

Flaxseed (linseed) is the richest source of lignans in the plant kingdom, the main component being SECO (over 360 000 µg/100 g dw) with minor amounts of MAT (Table 2). When flaxseed is crushed and defatted, SECO content rises up to 600 000–700 000 µg/100 g which yields absolutely the highest and unparalleled concentration of lignans from plant sources. These very high amounts of SECO in flaxseed are 4–5 times greater than concentrations reported by other investigators using other techniques, chiefly HPLC (85, 87, 99, 100). Significant differences in flaxseed lignan content have been observed among varieties (87) similarly such differences have been shown for soybean isoflavones (92). However, in this case it is more likely that methodological dissimilarities are responsible for the huge difference. In opposite to other methods our procedure can measure the dehydrated anhydroSECO product of SECO, which is formed during acid hydrolysis.

Table 1. Phyto-oestrogen content of food legumes (µg/100 g dry weight, ranges for species; method ID-GC-MS-SIM)

Botanical name Common name (number of cultivars)	DAIDZEIN	GENISTEIN	SECO	MAT
Glycine max Soybean (4)[a]	10 500–56 000	26 800–84 100	13–273	trace
Phaseolus vulgaris Kidney bean (11)	7–40	18–518	56–153	trace
Apios americana American 'Groundnut' (5)	0–18	108–811	21–58	2–5
Cajanus cajan 'Pigeon pea' (3)	12–27	190–737	19–50	0
Cicer arietinum Chickpea (3)[b]	11–192	69–214	7–8	0
Pisum sativum Pea (4)	4–11	0–23	3–13	0–trace
Vigna mungo Black gram (3)	7–36	trace–60	46–240	71–262
Vigna unguiculata Cowpea (2)	21–30	12–56	195–196	0
Lens culinaris Lentil (2)	3–10	7–19	0–7	trace
Pueraria lobata Kudzu (leaf)[c]	375	2520	476	trace
Pueraria lobata Kudzu (root) Japanese Arrowroot[d]	185 000	12 600	31	trace

[a]Additionally, contained formononetin 18–121.
[b]Additionally, contained formononetin 94–215 and biochanin A 838–3080.
[c]Additionally, contained formononetin 87, biochanin A 1240 and coumestrol 18.
[d]Additionally, contained formononetin 7090, biochanin A 1400 and coumestrol 1570.
trace—Present in trace amounts

Furthermore, our acid hydrolysis conditions (2 M HCl, 100 °C, 2.5 h), the step after enzymatic hydrolysis with Helix pomatia snail salivary gland extract, are more effective in liberating the lignan aglycones from their glycosidic forms than earlier methods. The substantial amount of MAT quantified is probably a result of the use of the more sensitive GC-MS-SIM detection system than in GC and HPLC methods.

We have analysed other oilseeds (13) and found significantly lower concentrations of lignans than in flaxseed (Table 2). Together with SECO and MAT in all oilseeds (except linseed) we determined daidzein and genistein. Nuts, containing substantial amounts of the lignan SECO (ranging 96–257 µg/100 g), are poor sources of isoflavones and MAT (Table 2). Oils derived from oilseeds (including soy oil) contain trace amounts of phyto-oestrogens (13).

Grains and cereals

In addition to their fibre, phytic acid and a variety of phenolic compounds (caffeic, ferrulic, gallic and ellagic acids), grains contain phyto-oestrogens. The literature does not contain phyto-oestrogen values of grains and their products. Recently we have analysed rye meal, rye grain milling fraction (101–102) and rye bread samples, and other cereals (13), and found substantial amounts of lignans SECO and MAT (range 8–132 µg/100 g and 0–167 µg/100 g dw, respectively), but no or trace concentrations of isoflavones (Table 3).

Table 2. Phyto-oestrogen content of oilseeds and nuts (µg/100 g dry weight, method ID-GC-MS-SIM).

Common name	DAIDZEIN	GENISTEIN	SECO	MAT
Flaxseed	0	0	369 900	1087
Flaxseed crushed	0	0	546 000	1300
Sesame seed	140	14	90	608
Clover seed[a]	178	323	13	4
Sunflower seed	8	14	610	0
Caraway seed	0	8	221	6
Poppy seed	18	7	14	12
Peanut[b]	58	64	298	trace
Cashew nut	0	0	257	4
Hazelnut	trace	trace	119	4
Pistachio nut	0	0	96	0
Walnut	5	trace	163	5
Almond	4	0	107	trace

[a] Additionally, contained formononetin 1270, biochanin A 381 and coumestrol 5.
[b] Additionally, contained biochanin A 31.
trace—Present in trace amounts

Using the GC-MS techniques we have observed that the lignans are localized in the outer fibre-containing layers with the highest concentration in the aleurone layer (3, 101) containing phytin, polyphenols, enzyme inhibitors and other compounds generally regarded as antinutritional factors. This 1 to 3 cell thick layer is tightly bound to the fibre layer, and liberation of the lignan precursors from these very resistant cells is difficult (103). Because of its close association with the outer fibre layer, modern milling techniques usually eliminate the aleurone layer which seldom, therefore, is present in commercial products, particularly in Western societies.

Table 3. Phyto-oestrogen content of grains and cereals (µg/100 g dry weight, method ID-GC-MS-SIM)

Common name	DAIDZEIN	GENISTEIN	SECO	MT
Wheat (whole grain)	0	0	33	3
Wheat white meal	trace	trace	8	0
Wheat bran	4	7	110	0
Oat meal	0	0	13	0
Oat bran	0	0	24	155
Barley (whole grain)	14	8	58	0
Barley bran	6	16	63	0
Rye meal (whole grain)	0	0	47	65
Rye bran	0	0	132	167
Triticale (whole grain)	2	2	39	9
Triticale meal	2	1	21	11

trace—Present in trace amounts.

Berries and fruits

As early as in the middle of 1980s, based on correlation found between dietary intake of berries and fruits fibre, and enterolactone and enterodiol urinary excretion, we suggested (93, 104–105) that berries and fruits contained plant lignans SECO and MAT, the precursors of mammalian enterolactone and enterodiol. Recently this observation has been confirmed with regard to berries. Lingonberry (the integral part of a traditional Finnish rye porridge meal) contains up to 1500 µg/100 g dw of SECO, followed by strawberry and cranberry with 1200 µg/100 and 1050 µg/100 g dw of this lignan (Table 4), respectively. There is a ten-fold difference in lignan concentration between the high- and low-content berries

(raspberry and red currant, respectively). It is of interest that the berries contain no isoflavones or MAT (with exception for trace amounts in strawberry and black currant).

Until now we have analysed only a few fresh fruits (Table 4) though the preparation procedures (collection of fruits and dry weight determination) for others have been accomplished. Based on results for apple, banana and plum we judge that these fruits contain minimal concentrations of both the lignans and the isoflavones. However, results of studies on urine of subject consuming exotic stone fruits (papaya, kiwi, guava, passion fruit) suggest that these fruits may contain a lot of isoflavones (W. Mazur, unpublished).

Table 4. Phyto-oestrogen content of berries and fruits (µg/100 g dry weight, method ID-GC-MS-SIM)

Common name	DAIDZEIN	GENISTEIN	SECO	MAT
Lingonberry	0	0	1510	0
Strawberry	0	0	1205	5.0
Cranberry	0	0	1054	0
Blueberry	0	0	835	0
Black currant	0	0	388	10
Cloudberry	0	0	203	0
Raspberry	0	trace	139	0
Red currant	0	0	165	0
Apple	12	0	trace	0
Sharon[a]	0	0	145	0
Banana	0	0	10	0
Plum	0	0	5	0

[a]Additionally, contained formononetin 12.
trace—Present in trace amounts

Cruciferous vegetables

Broccoli, cauliflower, cabbage, or Brussels sprouts contain several potentially anticarcinogenic bioactive microconstituents (i.e. dithiolthiones, isothiocyanates, and indole-3-carbinol precursors) and their consumption has been associated with a reduction in cancer incidence. Indole-3-carbinol has been revealed to exhibit anti-oestrogenic activity and inhibition of mammary cancer in rodent models (106). Results of our analytical studies yielded surprisingly low levels of isoflavones in fresh Cruciferous vegetables (13). These plants contain small amounts of daidzein and genistein ranging from 0 to 6 µg/100 g and 5 to 14 µg/100 g dw of daidzein and genistein, respectively (Table 5). Lignan SECO levels, however, were higher and varied from 33 µg/100 g in cabbage to 414 µg/100 g dw in broccoli. MAT was detected in broccoli whereas in the other Cruciferous plants analysed by us it was found only in trace amounts.

Allium Vegetables

Garlic and onion have been indicated to reduce risk of stomach cancer (107) and cardiovascular disease (108). The beneficial components are thought to be diallyl sulfide and allyl methyl trisulfide. We have analysed garlic and onion (Table 5) and found SECO (380 and 83 µg/100 g dw, respectively) but no isoflavones (13).

Other vegetables

In general, vegetables do not contain isoflavones. However, along with vitamins, chlorophyll, fibre, carotenes, aliphatic sulfides, aromatic isothiocyanates, phytic acid and other plant phenolics, they contain high levels of lignan SECO. Concentrations of SECO varies from 10 µg/100 g dw in potato to

817 µg/100 g dw and 3870 µg/100 g dw in zuccinia and pumpkin (peeled), respectively, with average amount exceeding 100 µg/100 g dw (Table 5). MAT, which increases in plants under fungal attack (109), were found in minimal amounts.

Table 5. Phyto-oestrogen content of vegetables (µg/100 g dry weight, method ID-GC-MS-SIM**)**

Common name	DAIDZEIN	GENISTEIN	SECO	MAT
Cabbage	trace	trace	33	trace
Turnip-rooted cabbage	0	5	38	trace
Red cabbage[a]	5	14	141	trace
Broccoli[b]	6	8	414	23
Cauliflower	5	9	97	trace
Onion	0	0	83	8
Garlic	0	0	380	trace
Witloof	0	0	175	15
Zuccinia (with peel)	0	0	817	trace
Pumpkin (peeled)	0	0	3870	4
Carrot (with peel)	0	0	370	trace
Paprika	0	0	117	7
Beetroot	0	0	100	trace
Potato (peeled)	0	0	10	6

[a] Additionally, contained formononetin 11.
[b] Additionally, contained coumestrol 8.
trace—Present in trace amounts

Beverages

Tea has been revealed to contain numerous polyphenols (flavanols /catechins/, flavonols, flavonediols, and phenolic acids, bisflavonols, theaflavins, thearubigens, and other oligomers), which may have both beneficial and hazardous effects in the human. Recently, using our GC-MS method we have examined over 20 black, green and oolong tea samples and for comparison 6 samples of coffee (110).The analysis of the teas yielded relatively high levels of the lignans SECO (561–2890 µg/100 g) and MAT (56–413 µg/100 g) but only low levels of isoflavonoids. For a part of the tea samples, brewing in hot water as the first step of the method was employed. The most important observation is that the lignans are almost quantitatively liberated by this process into the water from the green teas, but only partly from the black teas. Coffee meal samples processed according to the original method were shown to contain 2–3 times less of lignans, SECO (393–716 µg/100 g) and no MAT. Lignan results for selected teas are presented in Table 6.

Biochanin A has been detected in bourbon (111), genistein and daidzein in beer (112). After the development of radioimmunoassays for daidzein, genistein, formononetin and biochanin A in a collaborative study, all four isoflavonoids were chromatographically identified in beer and their concentrations determined (113–115).

DISCUSSION

Dietary phyto-oestrogens have shown interesting activities suggesting that they may offer protection against a wide range of human conditions, from hormone-dependant breast, prostate, and bowel and other cancers, cardiovascular disease, osteoporosis, and menopausal symptoms. The nature of this protection seems to be a result of a number of different mechanisms and interactions with endogenous factors like steroid hormones. Most of the mechanisms still remain unknown.

Table 6. Phyto-oestrogen content of black and green teas (μg/100 g dry weight, method ID-GC-MS-SIM)

Tea brand (origin)	Tea leaves Standard method		Tea brewed The aqueous infusion analysed	
Black/Green teas*	**SECO**	**MAT**	**SECO**	**MAT**
Pure Lapsang Souchong tea (China)	2130	250	1050	90
Earl Grey tea (Oriental tea mixture)	1259	197	1650	106
Yellow label tea-bag (not known)	1250	157	1092	92
Prince of Wales, tea-bag (not known)	2632	413	2418	305
China Green tea	2702	181	2887	195
Nippon Sencha Green tea	2466	186	2701	212
China Gunpowder	1491	178	1794	209
Japan Sencha Green tea	1648	263	1887	277

*Additionally, most of the black and green teas contained small amounts of isoflavonoids formononetin, daidzein, biochanin A and genistein—ranging from 5 to 78 μg/100 g.

One of the fundamental issues in the field of phyto-oestrogens deals with screenings of the most commonly consumed plants and plant-derived foods. Our ID-GC-MS method has allowed a specific, quantitative, reproducible and sensitive determination of the biologically most important isoflavones and lignans in food samples. The method has been applied successfully to a wide-spectrum of edible plants from those devoid of phyto-oestrogens to those containing high concentrations of the chemicals. Although the isoflavones are restricted within the Leguminosae, the lignans, particularly SECO, are wide-spread in plant foods.

The results of our studies demonstrate that plants, besides a large diversity of chemicals with a broad spectrum of biological properties, contain biologically active phyto-oestrogens—precursors of hormone-like compounds in mammalian systems.

Our recent experiments on rye-derived lignans and their metabolism in pigs indicate, however, that a large amount of lignans may be underestimated or that most of the mammalian lignan precursors are still unidentified (unpublished observation). Dietary lignans may be protected from analytical hydrolysis by the surrounding plant cell wall (which comprise the majority of dietary fibre in cereals) or by other components of food matrix. They may also be underestimated due to the presence of particular lignan structures, for example lignan polymers as suggested (116), which may escape analytical determination and still be degraded and available in the body. However, for flaxseed this problem does not seem to exist.

It has been hypothesized that environmental and dietary oestrogens (xenohormones) may be associated with the increased incidence of breast cancer in women and decreased sperm concentrations and reproductive problem in men. However, in light of the most recent reports (117–118) this is not the case for beneficial xeno-oestrogens, such as isoflavones and other bioflavonoids that occur in legumes, vegetables, fruits and grain products. It has been demonstrated (119) that phyto-oestrogens, unlike the natural oestrogens like oestradiol and some xeno-oestrogens, are devoid of a lipophilic region that may modulate binding affinities for these compounds at different ligand-binding sites (e.g. oestrogen receptor or oestrogen-metabolizing enzymes), and in fact may differentiate harmful from beneficial oestrogens. These results suggest that the proposed linkage between isoflavones and lignans, and breast cancer does not hold true, and further research is needed to determine other factors associated with the increasing incidence of this disease. On the contrary, the phyto-oestrogens, due to their biological properties, may have great potential in prevention against two scourges of mankind, hormone-dependant cancer and heart disease. There is already some relatively strong evidence indicating that low enterolactone levels in plasma or urine is associated with high breast cancer risk (1,120,121).

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