PHYTOESTROGENS: OCCURRENCE IN FOODS, AND METABOLISM OF LIGNANS IN MAN AND PIGS

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Helsinki 2000

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ACADEMIC DISSERTATION

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“Whoever gives these things [food] no consideration and is ignorant of them, how can he understand the disease of man?”

Hippocrates, 460-377 B.C.

To Ewa

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ABBREVIATIONS

AX	arabinoxylans
CHD	coronary heart disease
DAD	diode array detector
DW, dw	dry weight
END	enterodiol
ENL	enterolactone
ER	estrogen receptor
GC	gas chromatography
HP	Hewlett-Packard
HPLC	high-performance liquid chromatography
HRT	hormone replacement therapy
ID-GC-MS-SIM	isotope dillution gas chromatography - mass spectrometry in selected ion monitoring
MAT	matairesinol
NSP	non-starch polysaccharides
O-DMA	*O*-desmethylangolensis
SECO	secoisolariciresinol
TR-FIA	time-resolved fluoroimmunoassay
TFA	trifluoroacetic acid
TMS	trimethylsilyl
UV	ultra violet

LIST OF ORIGINAL COMMUNICATIONS

The thesis is based on the following papers, which are referred to in the text by roman numerals I – VI in parentheses, e.g. (I).

I Mazur, W., Fotsis, T., Wähälä, K., Ojala, S., Salakka, A. & Adlercreutz, H. (1996). Isotope dilution gas chromatographic-mass spectrometric method for the determination of isoflavonoids, coumestrol, and lignans in food samples. Anal Biochem, **233,** 169-180.

II Mazur, W., Wähälä, K., Rasku, S., Salakka, A., Hase, T. & Adlercreutz, H. (1997). Lignan and isoflavonoid concentrations in tea and coffee. Br J Nutr, **79,** 37-45.

III Mazur, W.M., Duke, J.A., Wähälä, K., Rasku, S. & Adlercreutz, H. (1998). Isoflavonoids and lignans in legumes: nutritional and health aspects in the human. J Nutr Biochem, **9,** 193-200.

IV Mazur, W.M. & Adlercreutz, H. (1998). Naturally occurring oestrogens in food. J Pure Appl Chem **70,** 1759-1776.

V Mazur, W., Uehara, M., Wähälä, K. & Adlercreutz, H. (1999). Phytoestrogens in berries - plasma and urine enterolactone concentrations after a single strawberry meal. Br J Nutr, **in press**.

VI Glitsø, L.V., Mazur, W., Adlercreutz, H., Wähälä, K., Sandström, G. & Bach Knudsen, K.E. (2000). Intestinal metabolism of rye lignans in pigs. Br J Nutr, **in press**.

INTRODUCTION

The principal killer diseases of our time, atherosclerotic vascular disease and cancer, are both chronic degenerative conditions of multiple aetiology. It is now apparent that, although genetic predisposition varies, the main factors determining whether or not a person becomes afflicted with these diseases are environmental. Diet is undoubtedly a key factor involved in the aetiology of both atherosclerosis and cancer, and its role may depend on the balance of nutrient and non-nutrient interactions. The individual's response to these dietary factors is in turn determined by genetic, physiologic and life-style factors.

Numerous epidemiological studies have shown that diets low in fat and rich in complex carbohydrates from vegetables, fruits and grains are associated with decreased risk of chronic diseases (Doll & Peto 1981; Trowell & Burkitt 1981; Steinmetz & Potter 1991a; Steinmetz & Potter 1991b; Dragsted *et al.* 1993; World Cancer Research Fund & Research 1997). Recent international epidemiological comparisons have linked the semi-vegetarian diet in some Asian countries with a reduced incidence of these diseases (i.e. the major hormone-dependent cancers, colon cancer, and coronary heart disease), indicating that some non-nutrient compounds in this diet may contribute to homeostasis and thus have a role in the maintenance of health. Lignans and isoflavonoids, recently detected and identified in human body fluids, are both of plant origin and have molecular weights and structures similar to those of steroids, implying that they could be such important dietary modulators of the human hormonal system (Adlercreutz 1990; Adlercreutz 1984; Bannwart *et al*. 1984a; Adlercreutz *et al*. 1986d; Setchell *et al*. 1984). The plant lignan and isoflavonoid glycosides are transformed by intestinal bacteria to hormone-like compounds (Setchell *et al*. 1981; Axelson & Setchell 1981; Setchell *et al*. 1982; Borriello *et al*.

1985). The mechanisms through which the phytoestrogens may influence sex hormone production, metabolism and biological activity could depend, at least in part, on their mixed estrogen agonist/antagonist properties and binding to estrogen receptors. Furthermore, these weakly estrogenic molecules have been demonstrated to affect intracellular enzymes, protein synthesis, growth factor action, malignant cell proliferation, cell differentiation, cell adhesion, angiogenesis, and apoptosis. Experimental studies in animals suggest that both lignans and isoflavonoids are among the dietary factors affording protection against atherosclerotic vascular disease and cancer (Adlercreutz & Mazur 1997; Clarkson *et al*. 1995; Murkies *et al*. 1998; Tham *et al*. 1998; Bingham *et al*. 1998; Lamartiniere *et al*. 1998; Thompson 1998; Barnes 1998).

In vitro and *in vivo* animal studies have revealed interesting properties of phytoestrogens and food containing these compounds by showing that they inhibit carcinogenic and atherosclerotic processes. However, many fundamental questions remain to be answered. Very little is known about the intake of phytoestrogens in various populations in relation to the risk of disease. In view of the chemoprotective role of these non-nutrients, it is of importance to know more about their presence and concentrations 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 dietary components, such as phytoestrogens, in foods. Lack of reliable assays allowing the phytoestrogen composition of food to be determined has hindered progress. Lignans and isoflavonoids are not listed along with nutrients in tables of food composition, neither is it possible to calculate their intake.

In view of this deficiency in the field of phytoestrogen research the main objective of the present study was first to develop a specific, sensitive and reproducible quantitative assay to

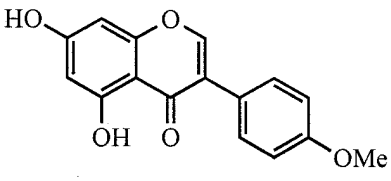
allow identification of foods that contain phytoestrogens. This, in turn, made it possible to assess the intake of the precursors of the biologically active compounds detected in the biological fluids of man. Hence all biologically important isoflavonoids and lignans in foods and diets were assayed using the method developed, allowing tabulation of the phytoestrogen composition of different plant food groups. In the third part of the project the assay method and other analytical procedures were applied to studies of bioavailability and metabolism of phytoestrogens in human subjects and in an animal model.

REVIEW OF THE LITERATURE

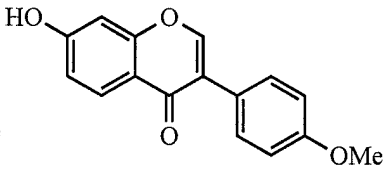
1. PHYTOESTROGENS - ESSENTIAL ISSUES

1. Definition, origin and classification

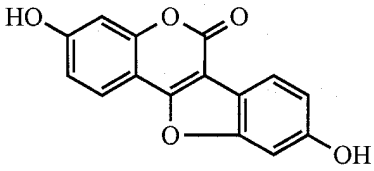
Dietary phytoestrogens are naturally occurring constituents of plants that elicit estradiol-like effects in one or more target tissues in animals. The classical definition of phytoestrogens encompasses compounds that exert estrogenic effects on the CNS, induce estrus, and stimulate growth of the genital tract of female animals (Lieberman 1996). Defined broadly, the term phytoestrogen refers also to chemicals that show effects suggestive of estrogenicity, such as binding to the estrogen receptors, induction of specific estrogen-responsive gene products, and stimulation of estrogen receptor(s)-positive breast cancer cell growth. In 1954 a list of 53 plants that possessed estrogenic activity was published (Bradbury & White 1954). A comprehensive review (Farnsworth *et al*. 1975) later expanded this list to over 300. From among the diversity of 'plant estrogens', isoflavones and coumestans (Fig. 1) have been identified as the most common estrogenic compounds in these plants (Price & Fenwick 1985) and have been given the name phytoestrogens. Plant lignans, precursors of weakly estrogenic compounds in mammalian systems (Welshons *et al*. 1987), have now been included among the phytoestrogens. The majority are diphenolic (better bisphenolic) compounds with structural similarities to natural and synthetic estrogens and antiestrogens. Resorcylic acid lactones (e.g. zearalenone) were also included in the group due to their reported estrogenic effects. However, they are not intrinsic components of food plants but are secondary mould metabolites of fungal species and therefore should be defined as fungal estrogens.



Biochanin A
Mol. Wt.: 284



Formononetin
Mol. Wt.: 268



Coumestrol
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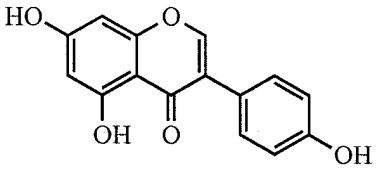
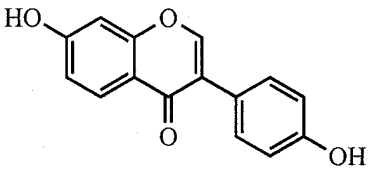
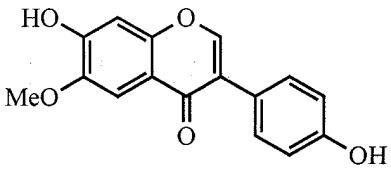


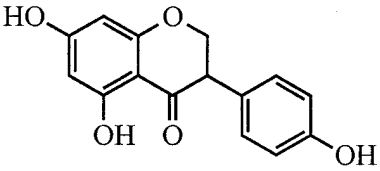
Genistein
Mol. Wt.: 270



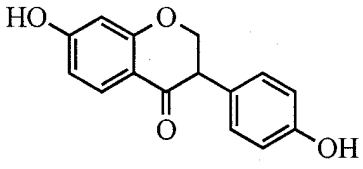
Daidzein
Mol. Wt.: 254



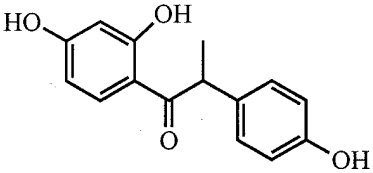
Glycitein
Mol. Wt.: 286



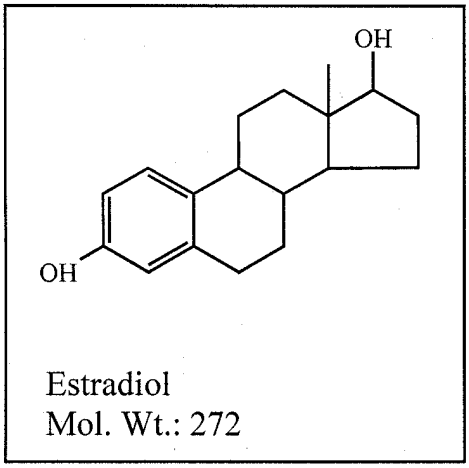
Dihydrogenistein
Mol. Wt.: 272



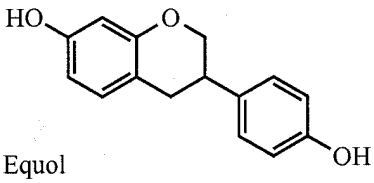
Dihydrodaidzein
Mol. Wt.: 256



O-desmethylangolensin
Mol. Wt.: 258



Estradiol
Mol. Wt.: 272



Equol
Mol. Wt.: 242

Fig. 1. The structures of the isoflavonoids (the main plant isoflavones and their mammalian metabolites), and of coumestrol, and the structure of estradiol.

1.2. Chemistry, estrogenicity and antiestrogenicity

1.2.1. Isoflavonoids.

Isoflavonoids (Fig. 1) comprise a large and very distinctive subclass of flavonoids. They encompass several structurally and biosynthetically related classes such as flavones, flavonols (3-hydroxyflavones), anthocyanins, flavanones, isoflavonoids (isoflavones, coumestans), and chalcones. Isoflavonoids differ structurally from other classes of flavonoids in having the phenyl ring (B-ring) attached at the 3- rather than at 2-position of the heterocyclic ring. In addition, isoflavonoids differ on account of 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.

Isoflavones constitute the largest group of natural isoflavonoids, with about 364 aglycones (unconjugated forms) having been reported (Dewick 1993). In this subclass the most thoroughly investigated and interesting compounds with regard to estrogenicity are genistein, daidzein, biochanin A and formononetin. The scientific interest in these natural compounds was initiated by their deleterious hormonal action in grazing animals. The estrogenic activity of clover, one of the richest source of phytoestrogens, was first described over 50 years ago following the observation that sheep feeding on pastures that containing clover demonstrated hyperestrogenization and infertility (Bennets *et al*. 1946; Braden *et al*. 1967; Shutt & Braden 1968). Crucial in demonstrating that phytoestrogens share a common mechanism of action were studies in experimental systems in which phytoestrogens competed with radiolabeled estradiol

for binding to the estrogen receptor and elicited estrogenic responses in estrogen-responsive tissues and cells (Price & Fenwick 1985).

Genistein (4`,5,7-trihydroxyisoflavone) is the most active principle with the highest binding affinity for the estrogen receptor (Shutt & Cox 1972). Its methoxy derivative, biochanin A, does not bind to the estrogenic receptor but is estrogenic *in vivo* (Braden *et al*. 1967; Miksicek, 1994). Daidzein (4`,7-dihydroxyisoflavone) has a higher binding affinity for the estrogen receptor than its methoxy derivative, formononetin, but both are weak estrogens *in vivo* (Shutt & Cox 1972). Methylation could be the mechanism through which the estrogenic potency of isoflavones is reduced (Bickoff 1961). The differential potency between genistein and daidzein could instead be referred to the presence of the 5-hydroxyl group of genistein (Bickoff 1961).

Results of a recent *in vitro* study in human breast cancer cells (Zava & Duwe 1997) revealed that genistein had estrogenic and ER-independent cell growth-inhibitory actions. Over a physiologically relevant concentration range, genistein could serve both as a surrogate estrogen agonist and as a growth regulator. Miksicek (1995) suggested that an optimal pattern of hydroxylation seems to be necessary in order for a flavonoid to have estrogenic activity. He reported that those flavonoids with hydroxyl substituents at 4' and 7 positions were invariably estrogenic and that an additional hydroxyl group at the 5 position (e.g. genistein) increased estrogenic activity. Having more than four hydroxyl substituents (e.g. flavonol quercetin) or having a 4'-methoxylated substituent (e.g. hesperetin) appeared to abolish estrogenic activity of the flavonoid. A similar observation came from a recent site-directed mutagenesis study by Kao *et al.* (1998). The authors indicated that for ER, isoflavones were better ligands than flavones, and rings A and C of isoflavones were thought to mimic rings A and B of estrogens. This structural

dependence on a pattern of hydroxylation was recently reported in other studies (So *et al*. 1997; Le Bail *et al*. 1998), in which genistein acted as weak estrogen competing with 17ß-estradiol for binding to estrogen receptor-positive MCF-7 human breast cancer cells. By using a yeast estrogen system in which yeast cells were cotransformed with the human ER and two copies of an estrogen response element linked to the lacZ gene, Collins *et al.* (1997) recently examined the agonist/antagonist activity of various flavones and isoflavones. The IC_{50} values (i.e., the concentration of the ligand competitor at which the binding of radiolabeled 17ß-estradiol to the human ER was reduced to 50%) for coumestrol, genistein, biochanin A, chrysin and naringenin were determined to be 0.01, 2.0, 6.0, 33 and 45 μM, respectively. The results indicate that such isoflavones as genistein and biochanin A bind to ER 5-10 times stronger than such flavones as chrysin and such flavanones as naringenin.

Coumestans represent a fully oxidized version of the flavonoid pterocarpans and share the same systematic numbering. A number of coumestans have been identified from species of *Pueraria* and *Glycyrrhiza* (Dewick 1986; Dewick 1993). Their prominent and the most potent representative, coumestrol (3,9-dihydroxy-6H-benzofuro[3,2-c] [1] benzopyran-6-one) (Fig. 1), which is related to the coumarins, has been isolated from a few fodder and pasture plants (e.g. clover, alfalfa) belonging to the Leguminosae (Bickoff 1961; Bradbury & White 1954; Knuckles *et al*. 1976). Coumestrol has higher binding affinity for the estrogen receptor than genistein (Shemesh *et al*. 1972). This is consistent with the receptor binding model that appears to depend upon a phenolic group in the 4' position of isoflavones and in the 12' position of coumestans.

Estrogenic activity of glycitein in mice was reported in one study (Song *et al*. 1999). The data indicated that pure glycitein, when fed to female mice (3 mg/d, 4 days), exhibited weak

estrogenic activity, comparable to that of other soy isoflavones but much lower than that of diethylstilbestrol and 17ß-estradiol.

As opposed to their estrogenic effects, definite antiestrogenic effects of isoflavonoids have been observed *in vivo*, because the effects of synthetic or natural estrogens seem to be counteracted by administered isoflavonoids or by their presence in the diet (Folman & Pope 1969; Kitts *et al*. 1983). Isoflavonoids at concentrations of 100-1000 times higher than that of estradiol have been considered to be able to compete effectively with endogenous mammalian estrogens, bind to the ER, and prevent estrogen-stimulated growth in mammals (Adlercreutz *et al*. 1995a). Such high phytoestrogen levels are achieved by regular phytoestrogen consumption. On the other hand, equol, genistein and coumestrol have been found to act through estrogen receptor-mediated processes and do not show any antiestrogenic effects in human breast cancer cells in culture (Welshons *et al*. 1987; Mäkelä *et al*. 1994). It is doubtful whether any true receptor-mediated antiestrogenic effect of phytoestrogens at the cellular level exists, but other antiestrogenic mechanisms are possible.

Recent reports (Kuiper *et al*. 1996; Mosselman *et al*. 1996; Byers *et al*. 1997) identifying a novel rat ER ß and a subsequent study (Kuiper *et al*. 1997) of the ligand selectivities and the tissue distributions of both the ER subtypes α and ß have thrown new light on the estrogenic activity of phytoestrogens (Cassidy 1999). Both coumestrol and genistein exhibit a significantly higher affinity for ER ß protein than for ER α, which is interesting in the light of the high expression of estrogen ß mRNA in the secretory epithelial cells of the prostate, and in view of the prostate cancer protective properties that have been associated with these compounds. ER ß is expressed prominently in e.g. testicular tissue and in the cardiovascular system, and apparently

also in breast tumor cells (Dotzlaw *et al*. 1997). Interestingly, ER ß is found in brain, bone, bladder and vascular epithelia (Kuiper *et al*. 1997; Paech *et al*. 1997) tissues that are responsive to classical hormone replacement therapy (HRT).

1.2.2. Dietary and mammalian lignans.

Lignans are chemically related to polymeric lignins of the plant cell wall and are found mainly in woody tissues, where they form the building blocks for the formation of lignin (Ayres & Loike 1990). Lignans belong to a group of plant phenols whose structure is determined by the union of two cinnamic acid residues (2, 3-dibenzylbutane structure) or their biogenetic equivalents. They are assumed to be formed biosynthetically by stereospecific reductive coupling of phenylpropanoids such as coniferyl and sinapyl alcohols, but experimental evidence for such a pathway is still lacking.

The majority of the 200 or more naturally occurring lignans (Rao 1978; Ayres & Loike 1990) occur in the free state in heartwood tissue. Some have been isolated from other plant parts, such as root, leaf and flower, and in such cases they may be found as glycosides. Lignans occur widely in the wood of gymnosperm (e.g., pine) trees, and they have also been recorded in some 500 angiosperm families, where they have been found in both the wood and bark of the trees. Lignans are found throughout the plant kingdom; they have been isolated from over forty-six families, eighty-seven genera and one hundred and fifty species (Cole & Wiedhopf 1978). A register of natural lignans contains nearly five hundred of the compounds (Ayres & Loike 1990), although their number is constantly growing (Ward 1993). There is a review of lignans, neolignans and related compounds (Ward 1993), but it is mainly devoted to chemical problems such as

nomenclature, analysis and biosynthesis, and the distribution of lignans in the plant kingdom is not dealt with systematically.

Current research is focusing on a number of lignans recently identified in humans and several animals (Fig. 2). Both *trans*-2,3-bis(3-hydroxybenzyl)-γ-butyrolactone and 2,3-bis(3-hydroxybenzyl)-butane-1,4-diol, known respectively as enterolactone (ENL) and enterodiol (END) have been described as the major lignans present in serum, urine, bile, and seminal fluids of humans and animals (Setchell & Adlercreutz 1979; Stitch *et al*. 1980b; Setchell *et al*. 1980b). The mammalian-derived lignans differ from plant-derived (dietary) lignans in possessing phenolic hydroxyl groups only in the *meta* position of the aromatic rings. The dietary precursors of these lignans are secoisolariciresinol (SECO) /[(*R**,*R**)]-2,3-*bis*[(4-hydroxy-3-methoxyphenyl)methyl]-1,4-butanediol/ (Setchell *et al*. 1980a) and matairesinol (MAT) /[2*R*-*Trans*)-dihydro-3,4-*bis*[(4-hydroxy-3-methoxyphenyl)methyl]-2(3*H*)-furanone/ (Borriello *et al*. 1985).

Lignans structurally resemble synthetic estrogens and may function as weak estrogens or estrogen antagonists (Adlercreutz *et al*. 1987). However, experimental evidence concerning hormonal actions of lignans, as well as that of isoflavonoids, is conflicting. No detectable estrogenic activity of ENL and END was revealed in *in vivo* studies of mice (Setchell *et al*. 1981) although these lignans have been found to bind weakly to rat uterine cytosol (J.H. Clark and H. Adlercreutz, unpublished observation, 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 antiestrogen tamoxifen. No antiestrogenic properties were observed (Jordan *et al*. 1985). In another study, ENL inhibited *in vivo* estrogen-stimulated RNA synthesis in rat uterine tissue when administered 22 h before estradiol (Waters & Knowler 1982). The concentrations of

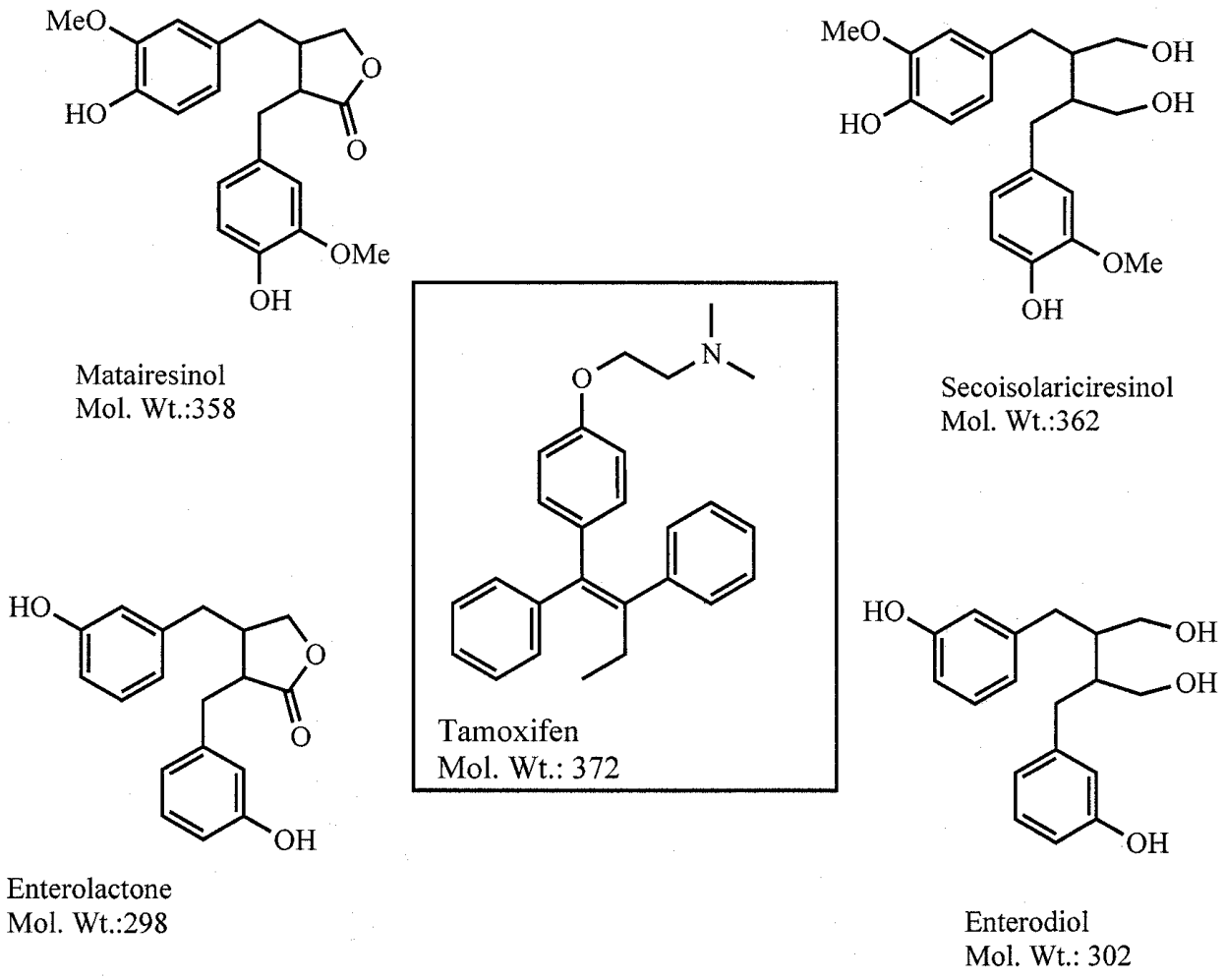


Fig. 2. The structures of the main dietary and mammalian lignans, and of the antiestrogen tamoxifen.

ENL were very low and it is doubtful whether this result can be repeated. A stimulatory effect of ENL on MCF-7 breast cancer cells in the absence of estradiol was observed, but a slightly stimulatory or nonstimulatory concentration of estradiol combined with a slightly stimulatory concentration of ENL did not cause stimulation or a tendency toward inhibition (Mousavi & Adlercreutz 1992). The ENL concentration was 1 μM, which could be regarded as physiological. ENL, but not END, was shown to stimulate pS2 expression in MCF-7 cells (Mäkelä *et al*. 1994; Sathyamoorthy *et al*. 1994). These diverging results are difficult to explain, but it has been suggested (Adlercreutz 1990; Whitten & Naftolin 1991) that the effect of exogenous weak estrogens may be either agonistic or antagonistic, depending on the level of endogenous estrogens. This has been experimentally confirmed with regard to coumestrol (Whitten & Naftolin 1991).

1.3. Metabolism and pharmacokinetics of phytoestrogens

In plants, phytoestrogens occur in different, at least fifteen, chemical forms. The isoflavonoids, like the lignans SECO and MAT, are conjugated with glucose (to form glycosides) and in soybean, the major glycosides are daidzin, genistin and glycitin. SECO-related lignans have also been reported to occur as polymers (Anderegg & Rowe 1974). The complexity of these substances is a major obstacle to the study of the metabolism of phytoestrogens in human subjects.

Isoflavonoids and lignans show similar patterns of metabolism in animals (Price & Fenwick 1985) and human subjects (Adlercreutz 1988; Adlercreutz *et al*. 1991c; Setchell & Adlercreutz 1988), while the metabolism of coumestans has not been characterized. When consumed by man, isoflavone and lignan glycosides are probably hydrolyzed in part by gastric

acid (Xu *et al*. 1995) and also undergo enzymatic hydrolysis by intestinal microflora. Intestinal bacterial glycosidases cleave the sugar moieties and release the biologically active aglycones which can be further biotransformed by bacteria to specific metabolites. The metabolism of formononetin, daidzein, biochanin A and genistein has been studied particularly in sheep (Nilsson *et al*. 1967; Braden *et al*. 1967; Shutt *et al*. 1970). Biochanin A is converted to genistein, which is further metabolized to dihydrogenistein, 6'-OH- desmethylangolensin and *p*-ethylphenol. *p*-Ethylphenol has not yet been identified in the human biological fluids. Most recently, based on the identification of metabolites from human urine, a more comprehensive metabolic pathway for daidzein was proposed by this laboratory (Heinonen *et al*. 1999). Formononetin is converted to daidzein, which is further metabolized to dihydrodaidzein, equol, *O*-desmethylangolensin (*O*-DMA) and *cis*-4-OH-equol. An intermediate-E (4',7-trihydroxyisoflav-2-ene) has been tentatively identified from human urine (Adlercreutz *et al*. 1987), but the full structural verification of this metabolite requires the synthesis of the reference compound. Equol was first identified as a minor constituent of the urine of pregnant mares (Marrian & Haslewood 1932) and later found in the urine of many other animal species (Braden *et al*. 1967; Klyne & Wright 1959; Knight & Eden 1995; Common & Aimsworth 1961; Shutt & Braden 1968), and of man (Axelson *et al*. 1982b; Axelson *et al*. 1984). Daidzein-7-ß-glucoside was isolated from soy flour and shown to be converted to equol glucuronide in man and rat (Axelson *et al*. 1984). Distinctively, mammalian derivatives of lignans (ENL, END) and isoflavones (equol) were not excreted in urine or found in the blood and bile of germ-free animals (Axelson & Setchell 1981), and administration of antibiotics prevented their formation in human subjects (Setchell *et al*. 1981; Adlercreutz *et al*.

1986c; Yasuda *et al*. 1994; Morton *et al*. 1994; Xu *et al*. 1995; Kurzer *et al*. 1995a; King *et al*. 1996; Sfakianos *et al*. 1997).

The metabolism of isoflavonoids varies from species to species and metabolism in man may therefore not be identical with that found in sheep or cattle (Braden *et al*. 1971). The hydrolysis of the flavonoid glycosides takes place in the proximal colon and several bacteria have been found which produce the necessary enzyme(s) (Bokkenheuser *et al*. 1987; Bokkenheuser & Winter 1988). The dominant position of lactobacilli, bacteroides and bifidobacteria among the intestinal flora indicates their major role in the intestinal hydrolysis of dietary ß-glycosides. After absorption in the small and probably large intestine, the isoflavones are transported to the liver, reconjugated and then excreted in urine and bile. No studies of the absorption of conjugated as compared to unconjugated isoflavones have been conducted in human subjects. Apparently, no attempts were made to determine the concentrations of isoflavone glycosides in plasma after high isoflavone-content meal, presumably because it is generally believed, although not proven, that intestinal hydrolysis of the glycoside is rapid and efficient. It is probably also difficult to separate glycosides from glucuronides, which are abundant in plasma.

Along with playing a central role in steroid-hormone metabolism, the liver is probably also important in the further metabolism of isoflavones and lignans by conjugating the aglycone with glucuronic acid and, to a lesser extent, sulfuric acid by hepatic phase II enzymes (UDP-glucuronosyltransferases and sulfotransferases) (Axelson & Setchell 1980; Morton *et al*. 1994; Adlercreutz *et al*. 1993). In plasma isoflavonoids and lignans occur as free compounds and as mono- and disulfates, and mono- and diglucuronides, and as sulfoglucuronides (Adlercreutz *et al*. 1993; Coward *et al*. 1996; Morton *et al*. 1994). As shown for Finnish postmenopausal

omnivorous and vegetarian women the free + sulfate fraction is low for genistein (3.8 % of total), but as much as 21-25 % of ENL and END occurs in this fraction (Adlercreutz *et al*. 1993). The efficiency of conjugation of isoflavones is high and consequently the proportion of circulating free isoflavones is small (Lapcík *et al*. 1998). The finding that ENL, END and equol were predominantly conjugated to glucuronic acid in portal venous blood (Axelson & Setchell 1980) suggested that conjugation of phytoestrogens may occur in the intestinal wall during absorption from the gut. This has been subsequently confirmed in rats by using averted intestinal sac preparations (Sfakianos *et al*. 1997). Lignans and isoflavonoids are mainly excreted as monoglucuronides in urine, but small amounts of diglucuronides and sulfates also occur (Axelson *et al*. 1982b; Bannwart *et al*. 1984a; Axelson *et al*. 1984; Axelson & Setchell 1980; Axelson & Setchell 1981). In faeces these phytoestrogens are excreted as free forms (more than 90 %) (Adlercreutz *et al*. 1995b).

In the course of gas liquid chromatography and mass spectrometry studies on steroid hormone metabolites in urine, new steroid-like compounds were identified and shown to belong to the chemical class referred to as lignans (Setchell *et al*. 1980a; Setchell & Adlercreutz 1979; Setchell *et al*. 1980b; Setchell *et al*. 1980c; Stitch *et al*. 1980b). Two major urinary lignans, revealed to possess a 2,3-dibenzylbutane structure, were given the names ENL and END. Of several monomethoxy and dimethoxy isomers of ENL and END detected (Setchell *et al*. 1980c; Setchell *et al*. 1981), MAT (Bannwart *et al*. 1984b) and SECO (Axelson *et al*. 1982a) were found to be intermediates in the pathway to the formation of ENL. When consumed, lignan glycosides are not, or poorly, absorbed from the small intestine because of their hydrophobic nature, and, being ß-glucosides, they are not easily hydrolyzed by mammalian enzymes, but readily by

bacterial enzymes (Rowland *et al*. 1999). It was observed very early that glucosides undergo a sequence of metabolic changes necessary for their conversion to mammalian lignans and subsequent absorption and utilization. The chemical reactions involve hydrolysis of the sugar moiety, dehydroxylation, demethylation and further oxidation. MAT glycoside is converted to ENL, and SECO diglycoside is transformed to END; the latter is further oxidized to ENL. The prerequisite for these reactions to occur is the presence of anaerobic microflora, which produce the required catalytic enzymes.

Lignans are closely related to phenolic estrogens (Adlercreutz & Mazur 1997) and therefore share the physiological features and behaviour of endogenous estrogens. The metabolism of sex steroid hormones involves an enterohepatic circulation that is dependent upon biologically active excretion, bacterial deconjugation, and intestinal reabsorption similar to that of the bile acids. Several decades ago the enterohepatic circulation of estrogens was studied in human subjects (Adlercreutz 1970; Eriksson & Gustafsson 1971; Adlercreutz & Martin 1980). In these studies it was estimated that about 50-60% of the conjugated estrogens in the liver are excreted into the bile and re-enter the human intestine. Deconjugation of the glucuronic and sulfuric acid conjugates, a step necessary for mucosal cell reabsorption, is catalyzed by the bacterial enzymes ß-glucuronidase and sulfatase, and is nearly complete. The finding of equol in high concentrations in portal venous blood of rats and in bile established enterohepatic circulation for isoflavones and lignans (Axelson & Setchell 1981). Recent studies of the pharmacokinetic behaviour of genistein in rats also confirmed that in common with endogenous estrogens (Adlercreutz 1970; Adlercreutz & Martin 1980) and flavonoids (Hackett, 1986), isoflavones undergo biliary excretion (Yasuda *et*

al. 1994; Sfakianos *et al*. 1997); infused genistein rapidly appears in bile. Direct evidence of the enterohepatic recycling of lignans in human has not been reported.

Most studies of the metabolism of phytoestrogens have focused on their urinary excretion. Human metabolism and excretion of isoflavonoids after soy (or soy products) consumption showed considerable individual variation (Hutchins *et al*. 1995b; Kelly *et al*. 1995; Xu *et al*. 1995). Early studies reported urinary equol excretion after a soy challenge (Axelson *et al*. 1984; Setchell *et al*. 1984) and urinary ENL excretion after a linseed challenge (Setchell *et al*. 1983b). Hutchins *et al.* (1995b) determined the presence of metabolites *O*-DMA and equol in addition to genistein and daidzein in urine after intake of fermented and unfermented soy foods, but no analyses were done in blood and faeces. They found out, however, that soy food processing appears to influence isoflavone bioavailability. King and his associates carried out two metabolic studies in rats and recently a third one in humans. The first rat study (King *et al*. 1996) reported the pharmacokinetics of pure genistein or genistein after administration of a soy extract and followed genistein concentrations in plasma, urine and faeces. In the other rat experiment (King 1998), comparing bioavailability of conjugates of soy isoflavones after a single dose of soy extract, plasma, urine and faecal daidzein and genistein and their metabolites (equol and 4-ethyl phenol) were investigated. In their human study (King & Bursill 1998) pharmacokinetics and urinary excretion patterns of the soy isoflavones daidzein and genistein were investigated. These measurements, however, included neither mammalian metabolites of the isoflavones nor faecal samples. Two pharmacokinetic studies have been conducted in which isoflavones were measured in man simultaneously in plasma, urine and faeces (Xu *et al*. 1994a; Watanabe *et al*. 1998). In the study by Xu *et al.* (1994a) genistein and daidzein, but no metabolites, were determined by HPLC.

The pharmacokinetic study of soybean (kinako) isoflavones conducted by Watanabe *et al.* (1998) is the most complete among metabolic studies of phytoestrogens. The authors reported a concentration of isoflavones in the kinako, basal levels of the isoflavones and their metabolites in plasma, urine and faeces, and further changes of concentration of the compounds in biological samples during 72-h period after a meal as measured by GC-MS. The plasma concentration of genistein increased after 2 h and reached its highest value of 2.44 +/- 0.65 μmol/L 6 h later. The plasma concentration of daidzein peaked at 1.56 +/- 0.34 μmol/L at the same time, but it was always lower than that of genistein. Peak plasma concentration of *O*-DMA and equol appeared after the daidzein peak in four and two subjects, respectively. In contrast to plasma, daidzein was the main component in urine. The half-lives of plasma genistein and daidzein were 8.36 and 5.79 h, respectively.

An interesting finding with regard to the metabolism of isoflavonoids in man was the observation that some people are unable to produce equol or that they excrete this isoflavone in very low amounts (Setchell *et al*. 1984; Axelson *et al*. 1984; Adlercreutz *et al*. 1991a; Xu *et al*. 1994a; Watanabe *et al*. 1998). Plasma equol was reported to be present in high concentrations in only 4 of 12 subjects challenged with soy flour (Morton *et al*. 1994). In a recent pharmacokinetic study, Watanabe *et al.* (1998) found individual variability in plasma and urinary concentrations of equol and *O*-DMA and classified the subjects as high and low metabolizers, re-affirming the original observations that about one-third of the general population cannot form equol from a dietary precursor daidzein (Setchell *et al*. 1984). This most recent study, as well as earlier one by Xu (Xu *et al*. 1994a), showed that the breakdown of isoflavones by the microflora in the gut determines the recovery of the compounds, and that the excretion to the urine of equol and *O*-

DMA is dependent on the different composition of intestinal microflora. Moreover, equol formation depends also on diet: a high fat/meat content diet increases equol production (Setchell & Adlercreutz 1988; Axelson *et al*. 1984). Such a correlation was also found for Japanese men and women consuming their traditional low-fat diet (Adlercreutz *et al*. 1991b); those consuming somewhat more fat and meat had significantly higher urinary excretion of equol than the other subjects. In contrast to this finding, Lampe et al. (1998) showed in women that equol excreters consumed a significantly higher percentage of energy as carbohydrate and greater amounts of plant protein and dietary fibre, both as soluble and insoluble fibre, compared to nonexcreters. Such differences were not observed in men, who overall had significantly higher fibre intakes than women. These data suggest that in women, dietary fibre or other components of a high-fibre diet may promote the growth and/or the activity of bacterial populations responsible for equol production in the colon. Accordingly, other studies (Wiseman 1999; Rowland *et al*. 1999) reported that good equol producers (35% of subjects and defined as > 1000 nmol/24 h) excreted about 200-fold more equol in their urine than the poor equol producers, and consumed significantly less fat and more carbohydrate, and also greater amounts of non-starch polysaccharides (NSP), compared with the poor equol producers. However, subjects who were good equol producers also had high levels of *O*-DMA, suggesting that, in those studies, equol and *O*-DMA did not appear to represent alternative pathways of daidzein metabolism (Rowland *et al*. 1999).

No age-related differences in isoflavone metabolism have been reported, although limited studies have examined plasma concentrations in young and old vegetarians and omnivores

(Adlercreutz *et al*. 1993), with no obvious difference in values found in the small sample sizes studied.

Recently, daidzein, genistein and glycitein bioavailability and metabolism were studied in urine and plasma of female and male subjects after consuming soymilk (high in genistein and daidzein) and soygerm (high in daidzein and glycitein) (Zhang *et al*. 1999). The average 48-h urinary excretion of glycitein, daidzein and genistein was similar to 55, 46 and 29% of the dose ingested, respectively. The excretion of these three isoflavonoids differed significantly from each other in men and women. Based on plasma isoflavone concentrations at 6 h after dosing, the bioavailabilities of daidzein and genistein were similar in men and women. At the high glycitein dose (soygerm), plasma concentration at 24 h after dosing suggested a modest gender difference in glycitein bioavailability.

The urinary excretion of lignans has been the most frequently studied aspect of their metabolism (Adlercreutz *et al*. 1982; 1986c; 1988; 1991b). Earlier studies in this laboratory have suggested that dietary fibre intake correlates with the urinary excretion of lignans (Adlercreutz 1984; Adlercreutz *et al*. 1981; 1982; 1986c; 1987), but also with the excretion of isoflavones (Adlercreutz *et al*. 1987). Statistically significant associations have been found between the intake of total fibre, fibre from berries and fruits, vegetable fibre, and legume fibre with the urinary excretion of these compounds (Adlercreutz *et al*. 1987).

Linseed has been used as a model to determine the *in vivo* metabolism and disposition of lignans and their precursors. Lignan availability after consumption of linseed has been investigated in some long-term studies in urine (Shultz *et al*. 1991; Lampe *et al*. 1994; Cunnane *et al*. 1995), and faeces (Kurzer *et al*. 1995a). In 19 premenopausal women consuming 10 g of ground linseed

per day for three menstrual cycles, both urinary (Lampe *et al*. 1994) and faecal (Kurzer *et al*. 1995a) lignan excretion increased and varied greatly among subjects (3- to 285-fold increase). Morton *et al.* (1994) reported plasma levels of lignans (and isoflavonoids) for postmenopausal women consuming a traditional diet supplemented with linseed, soy flour or clover sprouts. In another study, Morton et al. (1997b) fed four males with a cake containing 15 g cracked linseed and 15 g soybean flour and measured plasma concentrations of ENL (and daidzein and genistein) at short time intervals after consuming the meal. There was no increase in plasma ENL until about 8.5 h after consuming the cake. Brzezinski *et al.* (1997), studying the effects of a phytoestrogen-rich diet on menopausal symptoms in women, examined basal and end-of-study levels of phytoestrogens in serum without measuring the dietary content of plant estrogens. In none of these investigations were the basal and post-meal levels of lignans in blood and urine in human subjects measured after a challenge with food with known lignan precursor concentration. Practically nothing is known about the fate of lignans in common foods after ingestion in man. There is only one animal study in rats measuring food lignan content before intake and then following urinary levels of mammalian lignans (Landström *et al*. 1998).

Using GC-MS, Jacobs et al. (1999) has recently identified nine novel metabolites of ENL and END in the urine of female and male humans ingesting linseed for five days. The six identified metabolites of ENL were the products of monohydroxylation at the *para*-position and at both *ortho*-positions of the parent hydroxy group of either aromatic ring. Likewise, the three END metabolites were formed through aromatic monohydroxylation at the *para*- and *ortho*-positions. However, two of the six compounds (ENL metabolites) and one of the three (END metabolites)

may be intermediates of the bacterial conversion of SECO and/or MAT to ENL or SECO to END, respectively.

1.4. Physiological levels in human biological fluids

In this laboratory ID-GC-MS-SIM methods for the identification and quantitative determination of lignans and isoflavonoids in human urine (Adlercreutz *et al*. 1991a), plasma (Adlercreutz *et al*. 1993; 1994), faeces (Adlercreutz *et al*. 1995b) and amniotic fluid (Adlercreutz *et al*. 1999b) have been developed. A co-operative effort resulted in the development of analytical methodologies for the identification and measurement of phytoestrogens in human saliva, breast aspirate or cyst fluid and prostatic fluid (Finlay *et al*. 1991). A summary of all our results regarding urinary lignan and isoflavonoid excretion in various populations (Finnish women, American women, Asian immigrant women in Hawaii, breast cancer patients, and Japanese women and men) and dietary groups (omnivores, vegetarians, lacto-vegetarians, macrobiotics, and subjects consuming a traditional Japanese diet) has recently been presented (Adlercreutz *et al*. 1995a). Table 1 summarizes phytoestrogen levels in the biological fluids of various western and oriental populations derived from epidemiological studies (Adlercreutz *et al*. 1986a; 1994; 1991a; 1991b; 1995a; 1995b; Morton *et al*. 1997a).

1.5. Phytoestrogens and diseases

Strong and direct evidence of protective or anticarcinogenic effects of phytoestrogens and/or their metabolites *in vivo* in the human is still lacking. Epidemiological observations and laboratory animal and *in vitro* investigations have revealed a number of biological properties suggesting a

Table 1. Concentration of phytoestrogens in the biological fluids of different populations. [1]

	Biological fluid (units)	Daidzein	Genistein	Equol	*O*-DMA	Entero-lactone	Enterodiol	Mataire-sinol	Reference
Finnish									
Omnivores	Plasma (nmol/l)	4.2	4.9	0.8	0.07	29.0	1.4	0.02	(Adlercreutz 1994)
	Urine (nmol/24h)	133.5	184.4	37.5	1.45	2,350	161	nd	(Adlercreutz 1986b) (Adlercreutz 1991a)
	Feces (nmol/24h)	45.4	11.6	14.9	5.67	1,510	147	22.3	(Adlercreutz 1995b)
Vegetarians	Plasma (nmol/l)	18.5	17.1	0.7	0.8	89.1	5.4	0.06	(Adlercreutz 1994)
	Urine (nmol/24h)	94.0	nd	72.8	14.4	7,400	436	nd	(Adlercreutz 1986b) (Adlercreutz 1991a)
	Feces (nmol/24h)	259.1	189.6	257.8	114.7	3280	479.2	89.3	(Adlercreutz 1995b)
Breast cancer Patients [2]	Urine (nmol/24h)	182.0	nd	71.1	1.7	2,130	142	nd	(Adlercreutz 1995a)
American									
omnivores	Urine (nmol/24h)	372.0	nd	69.0	35.1	1,920	244	nd	(Adlercreutz 1986b)
macrobiotics	Urine (nmol/24h)	3,639	nd	817	44.8	19,900	7,096	nd	(Adlercreutz 1986b)
Japanese									
omnivores	Urine (nmol/24h)	2,580	4,910	250	350	500	270	10.0	(Adlercreutz 1991b)
British	Prostatic fluid								
omnivores	(nmol/l)	44.5	nd	2.1		68.0	8.6	nd	(Morton 1997)
Chinese	Prostatic fluid								
omnivores	(nmol/l)	95.7	nd	120.7		110	22.0	nd	(Morton 1997)

[1] Values are geometric means (urine) or mean values of total (plasma, faeces, prostatic fluid).

[2] Premenopausal women

nd - not determined

protective role for these compounds in a number of pathological conditions (Table 2). Special attention has been focused on hormone-dependent breast and prostate cancer, as well as on CHD. Table 3 summarizes potential biological effects exerted by phytoestrogens with regard to these diseases. Most recently the role of phytoestrogens in the genesis and prevention of chronic diseases was discussed in some reviews (Adlercreutz 1998b; Adlercreutz & Mazur 1997; Kurzer & Hu 1997; Bingham *et al*. 1998; Tham *et al*. 1998).

2. ISOFLAVONE CONTENT IN FOODS

2.1. Methods of analysis. The representatives of this class of flavonoids occur in foods almost entirely as complex mixtures of glycosides and glycoside esters (Bradbury & White 1954; Kudou *et al*. 1991b). Although only three carbohydrate conjugates of isoflavones have so far been identified, it is likely that in plants in general, a larger variety of glycosides exist. The analysis thus always requires complicated procedures including appropriate extraction and separation steps prior to final detection and quantification. Typically, in the case of conjugates, the isoflavones have been extracted from food (mostly soy) matrix with a hot aqueous polar solvent such as methanol or acetonitrile, either by simple mixing or by Soxhlet extraction (Eldridge 1982b; Farmakalidis & Murphy 1985; Jones *et al*. 1989; Wang *et al*. 1990; Kudou *et al*. 1991a; 1991b; Coward *et al*. 1993; Barnes *et al*. 1994; Kaufman *et al*. 1997). These methods led to the identification of the aglycones and the ß-glucoside conjugates as the principal components of the extracts. Other isoflavone glycosidic conjugates, such as 6’’-O-acetyl and -malonyl glucosides, were also identified (Ohta *et al*. 1979; 1980; Köster *et al*. 1983; Farmakalidis & Murphy 1985; Kudou *et al*. 1991a; 1991b). An easier way of analyzing the isoflavonoids is to hydrolyze the

Table 2. Phytoestrogens and prevention of Western diseases: potential health benefits.

Disease/pathology	**Potential health benefits**
• breast cancer • prostate cancer • hormone-dependent colon cancer • atherosclerosis • hypercholesterolemia • menopausal symptoms • osteoporosis • infections	• hormone-altering • estrogenic/antiestrogenic • antioxidative • cancerprotective • anticarcinogenic • antiproliferative • cardioprotective • antiatherogenic • hypocholesterolemic • antiosteoporotic • bone-maintaining • antiviral, antibacterial, insecticidal or fungistatic

Table 3. Biological effects exerted by phytoestrogens in principal chronic conditions of the Western world (Adlercreutz 1995a; 1998; Adlercreutz & M 1997)

BREAST CANCER	**PROSTATE CANCER**	**CORONARY HEART DISEASE**
Interference with release of gonadotrophins (prolonged menstrual cycle); in vivo Competition with endogenous estrogens for estrogen receptor binding (antiestrogenic effects); in vitro Interference with estradiol biosynthesis by inhibition of key steroid enzymes (steroid dehydrogenase, aromatase); in vitro Alteration of early mammary gland maturation in vivo in neonatal rats Antiestrogenic effects in vivo in sheep	Interference with androgen biosynthesis by inhibition of 5α-reductase; in vitro	Increase of vascular endothelial cell Ca^{2+} activated K^{+} channel currents; in vitro Inhibition of Na^{+}/H^{+} exchange induced by vasopress platelets; in vitro Cholesterol-lowering effects Increase of number of LDL-receptors; in vitro Inhibition of chemotaxis and cell cycle of vascular smooth cells; in vitro Inhibition of neointimal growth; in vitro Antioxidant effects on LDL, ex vivo
Stimulation of SHBG synthesis in the liver Inhibition of proliferation and protection of genetic damage; in vitro Inhibition of cellular protein tyrosine kinases (growth-inhibiting effects); in vitro Stimulation of differentiation; in vitro Immune-stimulating effects; in vitro Inhibition of topoisomerase I and II; in vitro		
Suppression of angiogenesis (retardation of endothelial cell growth) Antioxidative effects		

conjugates and monitor the formed aglycones. Partition between different solvents and enzymatic or acid hydrolysis of conjugated isoflavones has been used successfully in analytical studies of isoflavones (Naim *et al*. 1974; Seo & Morr 1984; Pettersson & Kiessling 1984; Setchell *et al*. 1987b; Wang *et al*. 1990; Cole & Cousin 1994; Liggins *et al*. 1998). The following enzymes have been used: various glucosidases (e.g. from almonds), *Helix pomatia* containing ß-glucuronidase, sulfatase and other enzymes, and cellulase from *Aspergillus niger*.

In most of the studies the isoflavones were separated by reversed phase HPLC using gradients mostly in mixtures of methanol or acetonitrile and aqueous acids or buffers. Setchell (1987b) compared sensitivity and reliability of UV, electrochemical and thermospray mass spectrometric detection following HPLC of soy-food extracts. Mass spectrometry in the selected ion monitoring mode has improved sensitivity one hundred fold over UV detection and has the advantage of improved specificity (Setchell *et al*. 1987b). Fluorescence (Wang *et al*. 1990; Kitada *et al*. 1985; Pettersson & Kiessling 1984) and electrochemical detection (Kitada *et al*. 1985; Setchell *et al*. 1987b) were shown to be useful in increasing the sensitivity of commonly used UV detection methods. Barnes *et al.* (1994) reported on the identification of isoflavones and their conjugates from soy products using HPLC mass spectrometry. Also in this laboratory in a number of soy products isoflavones and their glucosides, and lignans were analyzed applying the method (I) modified by protein precipitation with EtOH and using HPLC with coulometric electrode array detector (Nurmi & Adlercreutz 1999a). Most recently, Liggins et al. (1998) introduced a method for extraction and quantification of daidzein and genistein in foods using GC-MS-SIM, and Aussenac et al. (1998) presented results of capillary zone electrophoresis analysis of isoflavones in soybean extracts.

2.2. Isoflavones in foods. Although flavonoids are found in plants, vegetables and flowers in a bewildering display of biosynthetic prowess, isoflavonoids are found in just a few botanical families. The documented knowledge on isoflavonoid and lignan prevalence in plants is mostly dedicated to the chemistry of these compounds. Summarizing important developments in isoflavonoid chemistry of the period 1986-1990, P. Dewick wrote: "The isoflavonoids enjoy only a limited distribution in the plant kingdom, and are almost entirely restricted to the subfamily Papilionoideae of the Leguminosae. /.../ Among the non legume dicotyledons, a number of families are known to produce isoflavonoid derivatives, but only isolated plants or genera seem to have this ability, and the range of structures produced is very much more limited than in plants of the Leguminosae." (Dewick, 1993). The plant family most abundant in phytoestrogens is Leguminosae. Of all beans of the Leguminosae family the soybean and its products have attracted the most attention. Perhaps as a result of the economic importance of soybean and availability of research funds, most of the studies have been related to the isoflavones and soybean. Isoflavone content of soybean and numerous soybean-derived foods has been studied and reviewed extensively. Soybeans were first recognized to contain isoflavones over 65 years ago, when genistin was isolated in crystalline form from a 90% methanol extract of soybeans, and acid hydrolysis was shown to yield its aglycone, genistein (Walter, 1941; Walz, 1931). The first report presenting any quantitative data on the concentration of isoflavones in soybean was published by Naim et al. (1974). Since then, many other investigators have shown the abundance of isoflavones in soybean and other legumes (Murphy *et al*. 1982; Eldrige 1982a; Pettersson & Kiessling 1984; Setchell *et al*. 1987b; Coward *et al*. 1993; Wang & Murphy 1994; Barnes *et al*.

1994; Franke *et al*. 1995; Axelson *et al*. 1984; Fakutake *et al*. 1996; Kaufman *et al*. 1997). Studies have shown that there is a large variability in concentration and composition among different soybeans and soy-protein products (Murphy *et al*. 1982; Coward *et al*. 1993; Dwyer *et al*. 1994; Murphy *et al*. 1999; Franke *et al*. 1999) and that this is a function of species differences (Franke *et al*. 1995), geographic and environmental conditions (Eldridge & Kwolek 1983), as well as of the extent of industrial processing of soybeans (Seo & Morr 1984; Coward *et al*. 1993; Barnes *et al*. 1994).

Unfermented and fermented soy foods contain at least fifteen different chemical forms of isoflavones. Daidzein and genistein occur either as aglycones or as different types of glycoside conjugates. These include 6''-*O*-malonylglucosides, 6''-*O*-acetylglucosides, and the ß-glucosides of daidzein and genistein (Farmakalidis & Murphy 1985; Kudou *et al*. 1991b). Smaller amounts of glycitein conjugates are often found in soy proteins (Naim *et al*. 1973), whereas very high concentrations of conjugates of glycitein are found in the hypocotyledon or germ (Kudou *et al*. 1991b). The malonyl and acetyl glycosides are susceptible to heat and readily convert to the more stable ß-glycoside (Barnes *et al*. 1994); therefore, depending on the extent of processing of the soybean, the relative proportions of these conjugates can vary considerably among different soy foods (Coward *et al*. 1993; Wang & Murphy 1994).

Comprehensive lists of the isoflavone composition of many soy-protein foods of Western and Asian origin were published (Coward *et al*. 1993; Wang & Murphy 1994), and some additional studies have confirmed these findings (Farmakalidis & Murphy 1985; Dwyer *et al*. 1994; Franke *et al*. 1995). Recently a compendium of literature values of phytoestrogens, namely isoflavones and coumestrol, in foods was published (Reinli & Block 1996). This is a review of

different analytical procedures applied for analysis of soybeans, soybean products, soy foods and second-generation soy foods. Besides this variety of soy food derivatives, the authors reproduce HPLC results for various legumes published by Franke et al. (1994). Most recently Pillow et al. (1999) published the first report of a comprehensive database containing phytoestrogen values for foods included in a widely used, validated, food-frequency questionnaire. The phytoestrogens include isoflavonoids, coumestans, lignans, phytosterols, and flavonoids. Dietary lignan values presented in the database derive from our publications (I, II, III, Adlercreutz & Mazur 1997), and from a study by Obermeyer et al. (1995).

With regard to quantitative content of phytoestrogens in staple foods, the literature is scanty. There is only one study which has analyzed the isoflavonoid content in various foods other than soy (Jones *et al*. 1989). In their survey, Jones et al. (1989) could not detect any isoflavonoid levels in the 107 food items examined using HPLC system.

2.3. Lignans in foods. It has been suggested that the human diet, especially grain and other fibre–rich foods (Adlercreutz *et al*. 1981; 1984; 1986c), contains plant lignans, which act as precursors for the structurally modified mammalian lignans ENL and END, the modification being carried out by colonic microflora (Setchell *et al*. 1981; 1982; Axelson & Setchell 1981; Axelson *et al*. 1982a; Borriello *et al*. 1985). Adlercreutz et al. (1981; 1982; 1987; 1991b) have observed correlations between intake of plant foods and lignan excretion. A positive correlation between total fibre intake and urinary excretion of lignans was observed in premenopausal and postmenopausal vegetarian and omnivorous women living in Boston (USA) and Finland who were consuming their habitual diets (Adlercreutz *et al*. 1981; 1982; 1987). In addition, intake of

fibre from berries and fruits correlated positively with lignan excretion (Adlercreutz *et al*. 1987) and intake of energy from grain correlated positively with urinary ENL excretion (Adlercreutz *et al*. 1981). The urinary excretion of lignans ENL and END has been used in a clinical trial (Kirkman *et al*. 1995) to evaluate content of their precursors in foods. The results demonstrated that the excretion of mammalian lignans was higher during carotenoid (carrot and spinach) and cruciferous (broccoli and cauliflower) vegetable diets, than during a vegetable-free diet. In this study, men excreted more ENL and less END than women, implying a gender difference in colonic bacterial metabolism of lignans. In a similar well-controlled study, subjects, in response to alterations in vegetable, fruit, and legume intake, excreted in urine more END on the high vegetable/fruit diet than on the basal and legume/allium diets (Hutchins *et al*. 1995a).

Intestinal bacterial metabolism has profound effects on conversion, enterohepatic circulation and bioavailability of lignans. Administration of antibiotics almost completely eliminates the formation of ENL and END from plant precursors in the gut (Setchell *et al*. 1981; Adlercreutz *et al*. 1986a) and leads, after initial rapid lowering of the lignan levels in urine, to a relative increase in END/ENL ratio (Adlercreutz *et al*. 1986a). It has been suggested the waiting time before analyses can be carried out after a course of antibiotics should be at least 3 months (Adlercreutz 1998b) in order to obtain correct values reflecting dietary intake.

Evidence for dietary precursors was first obtained by changing the diet given to adult rats from commercial pellets to a semi-synthetic diet, which resulted in a marked and rapid decrease in the excretion of mammalian lignans in urine. When the diet was reverted to commercial pellets, lignans reappeared in the urine. The minimum concentration of lignan precursors in different food constituents was determined by adding the latter to the semi-synthetic diet and measuring the

total excretion of lignans in urine (Axelson *et al*. 1982a). In decreasing order, wheat bran, rye, buckwheat, millet, soy, oat and barley gave from 8 to 2 μg of lignans in urine per g of meal added. Linseed, however, was by far the richest source of precursors, giving about 800 μg per g of meal. Preliminary experiments to isolate the precursors from barley, rye, wheat bran and linseed revealed that the compounds were polar and could be extracted with 80% methanol and separated from the fibre. The plant lignans SECO and MAT, like other plant phenolics, could occur in plants as monomers or combine with other phytochemicals to form esters or glycosides. In an analytical study on linseed a "tan polymer" requiring methoxide treatment has been obtained (Bakke & Klosterman 1956). The presence of SECO polymers in the resin of parana pine knots has been reported (Anderegg & Rowe 1974).

Using another indirect technique, *in vitro* fermentation with human faecal microbiota (which simulated colonic fermentation), Thompson et al. (1991b) determined the production of mammalian lignans from 68 common plant foods. Amounts of mammalian lignans produced, measured using gas chromatography, ranged widely from 21-67,541 μg/100g sample. This study also found that linseed flour and its defatted meal were the highest producers of lignans. Vegetables, oilseeds, legumes, cereal bran, and whole grain cereals were reported to be good sources of lignans in an *in vitro* model. In a similar *in vitro* system (Nesbitt & Thompson, 1997), twenty-five foods, including raw linseed, homemade products containing linseed, and commercial breads and breakfast cereals with and without linseed, were subjected to a fermentation procedure designed to simulate the colonic environment necessary for the conversion of plant precursors to mammalian lignans. Lignan production was significantly related to the percentage of linseed in homemade products and breakfast cereals.

Results showing dietary lignan content assessed directly in plant foods have not been published (with the exception of the present study). Reinli & Block (1996) published a review of phytoestrogen content studies in 1996. Their compendium of literature values does not mention dietary lignans at all, probably because they have not usually been reported as phytoestrogens. Concentrations of lignans in various foods were tabulated in another large database study by Pillow et al. (1999). Isoflavonoids and lignans have never been measured in the same assay. In three studies (Obermeyer *et al*. 1995; Rickard *et al*. 1996; Thompson *et al*. 1997), however, separate measurements of lignans in linseed have been performed using HPLC with UV or MS detection as the determinative steps.

2.4. Glycitein and coumestrol in foods. Since glycitin and glycitein occur only in low or trace amounts in food, and particularly in soy foods (Kudou *et al*. 1991b), most studies have restricted measurements to the predominant analytes daidzein and genistein and their glycosides. Some studies included coumestrol (Eldridge 1982b; Murphy 1981; 1982; Setchell *et al*. 1987b; Jones *et al*. 1989; Wang *et al*. 1990; Franke *et al*. 1995). Apart from soy, coumestrol was found in alfalfa (Knuckles *et al*. 1876), lucerne (*Medicago sativa*), clovers and clover sprouts (Franke *et al*. 1994), and at low levels in beans and peas (Knuckles *et al*. 1876; Franke *et al*. 1994). Interestingly, coumestrol has been observed to accumulate in alfalfa following insect or fungal attack (Loper 1968). According to some studies, the coumestrol concentration of alfalfa is a consequence of fungal diseases (Smith *et al*. 1979) whereas in healthy plants it has been found in very small concentrations (Wong *et al*. 1971). These findings have not been confirmed by other investigators

of leguminous plants (Saloniemi *et al*. 1995). Recently, considerable amounts of coumestrol have been detected in edible soybean and alfalfa sprouts (Murphy 1982; Franke *et al*. 1995).

AIMS OF THE STUDY

The general purpose of the study was to assess human intake of phytoestrogens by measuring the content of isoflavones and lignans in selected foods common in Western and Oriental diets.

The specific aims of the study were:

1. To develop a specific, sensitive and reproducible quantitative method (isotope dilution gas chromatographic-mass spectrometric method; ID-GC-MS) to identify foods that contain phytoestrogens and to allow assessment of the intake of the precursors of the biologically active compounds detected in biological fluids.

2. To determine all biologically important isoflavonoids and lignans in foods and diets used in experimental studies using the ID-GC-MS method, and to compose tables of the phytoestrogen composition of the different food groups.

3. To study the pharmacokinetics of the chief mammalian lignan ENL in human subjects fed a meal with a known amount of plant lignan precursors.

4. To study the intestinal metabolism of plant lignans, their conversion to mammalian derivatives, and their bioavailability and excretion in the pig as a model of the human organism.

MATERIALS

1. Standards

1.1. Reference standards.

The dietary isoflavones formononetin, daidzein, genistein and biochanin A were synthesized by a *one pot* procedure starting from the appropriately substituted phenol and phenyl acetic acid in 53-99% yield (Wähälä *et al*. 1995). MAT was synthesized by a *one-pot* three-component Michael addition/alkylation sequence (Salakka 1996; Wähälä & Hase 1991). ENL and END were synthesized as described (Adlercreutz *et al*. 1986d).

1.2. Deuterated standards

d_4-Daidzein was prepared by H/D exchange in CF_3COOD, made from trifluoroacetic acid anhydride and D_2O under argon atmosphere in an isotopic purity of 90% or better (Wähälä *et al*. 1986; 1995). CF_3COOD deuterates readily the aromatic protons in isoflavones that are *ortho* or *para* to a phenolic hydroxy group (Wähälä 1997). Stable d_4-genistein and d_4-biochanin A were labeled by $D_3PO4_4 \cdot BF_3/D_2O$ (Rasku *et al*. 1999a). This deuteration method was also utilized to synthesize the stable d_3-formononetin and d_4-coumestrol (Rasku *et al*. 1999b). MAT was deuterated by D_3PO_4 (Adlercreutz *et al*. 1991a) or by $D_3PO4_4 \cdot BF_3/D_2O$ (Rasku *et al*. 1999a) to stable d_6-MAT in good yield and over 98% isotopic purity. Stable d_6-secoisolariciresinol was obtained by hydride reduction of d_6-MAT in 99% isotopic purity. The d_8-secoisolariciresinol was prepared from d_6-MAT using labelled hydride reagent (Rasku *et al*. 1999a). The stable d_8-SECO was converted under acidic conditions to d_8-anhydroSECO (Rasku *et al*. 1999a). d_6-ENL and d_6-END were synthesized as described (Wähälä *et al*. 1986).

2. Fluoroimmunoassay of ENL

Immunogen synthesis and immunization of rabbits, as well as labeling of the ENL derivative with europium chelate, were described in detail in papers presenting a time-resolved fluoroimmunoassy (TR-FIA) for plasma ENL (Adlercreutz *et al*. 1998a; Stumpf *et al*. 1999) and urine (Uehara *et al*. 1999), respectively.

3. Instrumentation

Capillary column gas chromatography: An HP 5890 Series II Gas Chromatograph combined with an HP 7673A Automatic Sampler and coupled with an HP data system consisting of an HP 7673A Controller, an HP 9114B Disk Station and an HP 3396A Integrator for processing of chromatographic data. The instrument was equipped with flame ionization detector (FID) and operated with helium as the carrier gas.

GC-MS instrument: (I, II, III) An HP 5995 quadrupole mass spectrometer with an Autoinjector 7673A and a data system HP 59970C MS Chem Station; and (IV, V) a Fisons MD 1000 quadrupole mass spectrometer utilizing a Fisons 8000 gas chromatograph and a Digital 5120 computer for processing of chromatographic and mass-spectrometric data. Helium was used as carrier gas.

The dry weight (dw) of dietary samples was determined with a Moisture Analyser 40 (Sartorius AG, Göttingen, Germany).

TR-FIA was done with a Victor 1420 multilabel counter using Wallac 1420 Victor software version 1.0 for data analysis, a 1296-026 DELFIA platewasher and a 1296-003 DELFIA

plateshaker. Radioactivity counting was performed with a LKB Model 1217 Rackbeta Liquid Scintillation Counter. All the instruments were purchased from Wallac, Turku, Finland.

4. Sources of samples

4.1. Development of the method and food analyses.

Samples analyzed contained common components of the human diet, including grains and cereal products, soybean and soy products, legumes seeds, nuts and seeds, vegetables, fruits and berries, and beverages such as tea, coffee and wine. Most of the samples were purchased from commercial sources except the 14-grain mixture used as a control sample and provided by Dr. Ernest Wynder (American Health Foundation, USA), and a few legumes kindly provided by Prof. Antony McMichael (London, UK).

In the human pharmacokinetic study, nine berries, the representatives of families *Rosaceae, Grossulariaceae* and *Ericaceae*, were analyzed for phytoestrogen contents. The berries were purchased from commercial sources. Strawberries for consumption by participants in the study were bought from a farm in central Finland. These strawberries were divided into 10 portions of 500 g (wet weight) each and frozen in -20°C until consumed. Before phytoestrogen analysis, the berry samples were dried in the oven and pulverized manually in a mortar. DW was determined prior to the analysis.

The pig diet samples, based on rye breads, were produced in an industrial bakery (Pandrup Brød, Schulstad A/S, Pandrup, Denmark) (Glitsø & Bach Knudsen 1999) in Denmark.

4.2. Human and animal studies

The pharmacokinetics of mammalian metabolites of berry-derived plant lignans were studied in seven apparently healthy volunteers (five females and two men; V). The subjects were included in the study based on several exclusion criteria (history of any serious disease, diabetes, renal or liver disease, regular consumption of alcohol or persistent food allergies). None were taking any medication and none had had any antibiotics during the 3 months preceding the sampling period. The subjects, age ranged from 23 - 55 (mv 38.1) and BMI from 18.8 to 26.0 (mv 23.3). The participants began a restricted diet (all food and beverages containing phytoestrogens were excluded) three days before the experiment (D 1) and followed it throughout the experiment. On day 3 (D 3), starting after the first morning void, participants collected one 24 h urine sample ("zero" urine), including the first morning void on day 4 (D 4). Daily urine was collected into 2-litre plastic bottles containing 2 g of ascorbic acid and stored at +4°C until the collection was completed. Total urine volume was measured and aliquots were stored at -20°C until analysis. On D 4, at 8 o'clock a.m., a vascular catheter was introduced into a median cubital (or basilic) vein and the first blood sample ("zero" plasma, 4 x 10 ml tube) was taken. Thereafter subjects consumed 500 g of strawberries. Following the strawberry meal the 24 h urine sample was collected in four portions: 4 h + 4 h + 4 h + 12 h. Blood samples (4 x 10 ml) were taken through the catheter at the following intervals: 0.5 h, 1 h, 2 h, 4 h, 8 h after the berry meal. The last blood sample was taken on the next day (D 5) in the morning (24 h after the meal). After centrifugation of the tubes, plasma (15 - 20 ml) was separated and stored at -20°C until analysis. On D 5 urine sample collection was continued in two portions (12 h + 12 h) per day. Total urine collection obtained in 4 (D 4) and 2 (D 5) portions was measured and stored in -20°C until analysis.

The intestinal concentrations and the disappearance of lignans were studied in twenty male castrated crossbreed pigs (5 pigs per diet; weight at surgery ≈ 30 kg; Danish Institute of Agricultural Sciences, Swine Herd, Foulum, Denmark; VI). The pig was chosen as a model of humans as it allows sampling of material from different parts of the intestinal tract opposed to collection of human faecal samples only. The digestive anatomy, physiology and metabolism in humans and pigs are comparable, even if the size and the capacity of the growing pig's digestive tract relative to body weight is greater than that of human adults. It is, however, agreed that among the species most extensively studied, the pig appeared to have a colon most similar to that of humans in regard to gross structure and absorptive characteristics, and the pig is generally regarded as a convenient and valuable model in investigations where human studies are not possible and *in vitro* techniques inadequate. The pigs were fitted with simple T-cannulas in the ileum approximately 15 cm anterior to the ileo-caecal junction and allowed to recover for 8-10 days. The pigs, after an adaptation period of 8-9 days on the experimental diets, were placed in metabolic cages (balance period) and faeces were collected for 72 hours. Following the faecal collection, ileal contents were sampled from the cannulas from 07.00 to 15.00 starting at the time of the morning feeding, and this was repeated two days later. The ileal collections were performed by attaching small plastic tubes to the cannula. The tubes were emptied either when they were full or they were emptied if they had been attached for 90 minutes. The pigs rested for a week, still consuming the experimental diets, and were then subjected to a similar second balance period in the metabolic cages. Three to four days after the last balance period the pigs were slaughtered 4 hours post-feeding by an overdose of pentobarbital. The gastro-intestinal tract was immediately

removed and intestinal material was collected from the caecum, the middle third of the colon, and rectum. Freeze-dried intestinal samples were analyzed for lignans.

5. Chemicals, reagents and preparation of glassware

Methanol, n-hexane of HPLC grade and analytical grade ethyl acetate were purchased from Rathburn Chemicals Ltd (Walkerburn, Peeblesshire, Scotland). Pyridine, toluene, glacial acetic acid of suprapure quality, sodium hydroxide, diethyl ether, ascorbic acid, hydrochloric acid were obtained from E. Merck AG (Darmstadt, Germany). Silyl 8, specially purified grade trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS) were obtained from Pierce Chemical Co. (Rockford, IL, USA). Dimethylchlorosilane (DMCS) was obtained from Fluka AG (Buchs, Switzerland) while *Siliconimprägnierer* was from Carl Roth GmbH (Karlsruhe, Germany). Charcoal (activated) was purchased from Sigma Chemical Co. (St. Louis, MO., USA) and *Helix pomatia* from Biosepra IBF/Sepracor (France). Charcoal was washed with water and dried.

All glassware including Pasteur pipettes was silanized with *Siliconimprägnierer* to avoid creepage of phytoestrogens and subsequent decomposition. The glass liner of the injection system of the GC-MS instrument was deactivated with 1% DMCS in toluene.

6. Anion exchange material

DEAE- and QAE-Sephadex A-25 exchangers in chloride form were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Before conversion to the appropriate form the gels were washed successively in 20%, 50% and absolute ethanol and then suspended and stored in methanol at

4°C. Conversion was carried out by washing the chloride form of the anion exchangers with 10 bed volumes of each of the appropriate solvents in a sintered glass funnel under vacuum suction. The hydroxyl form of DEAE-Sephadex was obtained by washing the gel (DEAE-Cl^-) with 0.1 mol/L sodium hydroxide in 70% methanol, 70% methanol, and methanol. The gel was used immediately after conversion. The acetate form of QAE-Sephadex was obtained by washing the gel (QAE-Cl^-) with 0.1 mol/L sodium hydroxide in 70% methanol, 70% methanol, 0.5 mol/L of acetic acid in 70% methanol, and 70% methanol. The acetate form of the exchanger could be stored in methanol at 4°C and used within 6 months after the conversion.

METHODS

1. Quantification of phytoestrogens in foods

Based on experience with ID-GC-MS methods for the identification and quantitative determination isoflavonoids and of lignans in human urine (Adlercreutz *et al*. 1991a), plasma (Adlercreutz *et al*. 1993; 1994) and faeces (Adlercreutz *et al*. 1995b), an original methodology for the quantitative determination of the phytoestrogens formononetin, biochanin A, daidzein, genistein, and coumestrol and simultaneously the lignans SECO and MAT in plant-derived foods was developed. These compounds were measured by ID-GC-MS in the SIM mode using stable synthesized deuterated internal standards for the correction of losses during the procedure. The method was applied to analyze a number of food and diet samples (including those used in the human and pig studies). All analyses were performed at least in duplicate on (freeze-) dried material (with some exceptions in II and IV). A flow diagram of the method is displayed in Fig. 3.

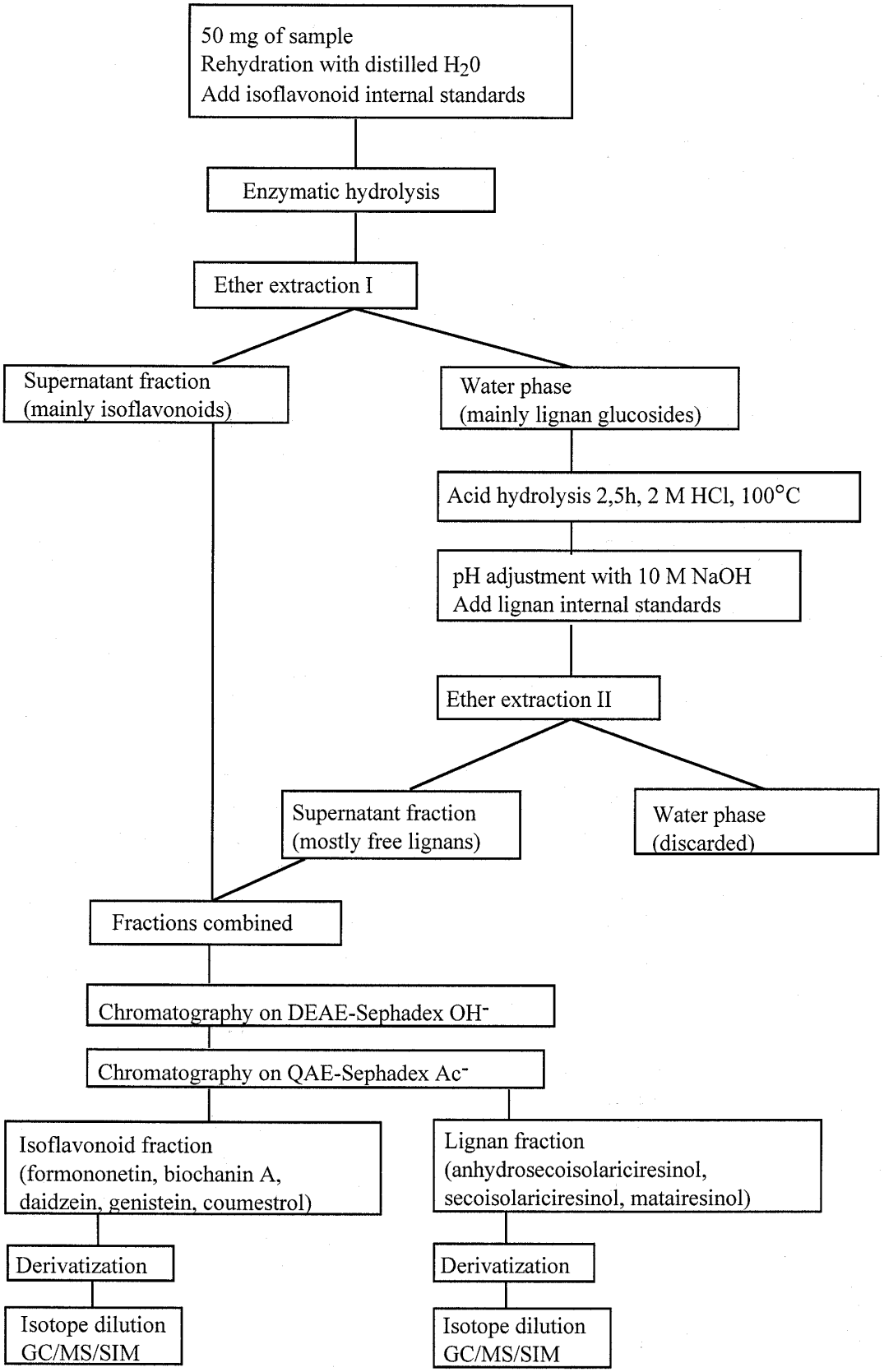


Fig. 3. Flow-diagram of the method used here to determine quantities of lignans and isoflavonoids in food samples.

1.1. Pretreatment of food/diet samples

Prior to the analysis the dry weight of samples was determined. Plant-derived or complex diet samples, unless received as freeze-dried, were dried in the oven (45-50°C, 4-5 days). The dried samples were ground with an electric grinder or pulverized with a mortar manually. All freeze-dried samples were ground to a particle size less than 0.5 mm prior to analysis (VI). The dry matter content was determined by drying the samples at 105°C for 20 hours (VI). A small amount of the milled or freeze-dried sample, usually 50 mg, was taken for analysis and rehydrated in a 15 ml glass tube with 500 µl of distilled water. The sample was left at room temperature overnight. For very fatty foods the water was then extracted with *n*-hexane (2 x 2.5 ml, vortexing 2 x 1 min) and the *n*-hexane was discarded.

1.2. Enzymatic hydrolysis

Isoflavonoid and lignan glycosides were hydrolyzed with *Helix pomatia* juice. *Helix pomatia* juice is a mixture of semipurified enzymes extracted from snail digestive juice and hepatopancreas. This solution has been standardized to contain 100,000 Fishman Units of ß-glucuronidase and 1,000,000 Roy Units of sulfatase per ml. Because the juice also contains small amounts of lignans and isoflavonoids which have to be removed, it was purified before usage by shaking with 1 % charcoal in 9 ml of 0.66 M acetate buffer (pH 4.1) overnight in a Shaker incubator SI 100 (Janke & Kunkel, Staufen, Germany) at room temperature. On the next day the purified *Helix pomatia* was centrifuged 10 min at 15,000 rpm with a Hettich Microliter centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). The supernatant was collected and re-centrifuged under the same conditions (10 min, 15,000 rpm) and then used for the assay.

Before hydrolysis the deuterated internal standards of formononetin, biochanin A, daidzein, genistein and coumestrol were added in amounts suitable for the expected concentrations of diphenols in the food sample as well as 2.5 mg of ascorbic acid for protection of labile natural compounds. Hydrolysis was carried out by adding 500 µl of 0.3 M acetate buffer, pH 4.1 containing 2,500 Fishman Units and 25,000 Roy Units of the purified *Helix pomatia* juice. The sample was gently mixed with a Vortex mixer and incubated in a Dri-Block (Techne Ltd., Cambridge, UK) for 2 h at 60°C. After the hydrolysis the sample was allowed to cool to room temperature.

1.3. Extraction

Free hydrolyzed isoflavonoids and lignans were extracted from the matrix with diethyl ether. Five ml of cold diethyl ether was added to about 1 ml of the hydrolyzed sample and gently mixed in a Vortex mixer for 2 min to avoid emulsification. Separation of ether and water phases was achieved by freezing in ethanol-solid carbon dioxide bath. The procedure was repeated. The two ether extracts were combined and evaporated to dryness under nitrogen with gentle heating (maximum 55°C) in a water bath. Finally the dry sample was redissolved in 0.5 ml methanol and stored in 20°C until combined with the fraction containing mainly lignans (see below). The traces of ether in the water phase were evaporated and the water phase was then subjected to acid hydrolysis.

1.4. Acid hydrolysis

The water phase was combined with 500 µl of 6 M hydrochloric acid to obtain the final concentration of 2 M. Then the sample was mixed with a Vortex mixer and incubated at 100 °C in

the Dri-Block. It was occasionally shaken in order to remove condensed water from the walls of the vial. After 2.5 h the sample was taken out and allowed to cool to room temperature. Finally pH was adjusted to 3-5 with 10 M sodium hydroxide (about 300 µl). Following partial neutralization of the extract, synthesized stable deuterium-labelled MAT, SECO and anhydroSECO (most of SECO present was converted quantitatively to this compound during acid hydrolysis) were added. Thereafter the sample was extracted with diethyl ether as described. This fraction, containing mainly lignans, was combined with the isoflavonoid fraction in methanol (see above). Finally, after evaporation of the solvent, the dry sample was redissolved in 0.5 ml of methanol and transferred to the DEAE-Sephadex-OH^- column.

In the pig study (VI) all raw material and dietary samples were also hydrolyzed with trifluoroacetic acid (TFA) (instead of HCl in the acid hydrolysis step) in a parallel experiment in order to control for results.

1.5. Ion exchange procedures

The GC-MS-SIM method is the only one developed for food samples that utilizes Sephadex gel chromatographies. The concept of using ion exchange chromatography in phytoestrogens analysis is based on anion exchange chromatography of steroid hormones. As early as 1956 Sjöström and Nykänen separated estradiol benzoate from testosterone, and in 1962 Hähnel was able to separate estrogen conjugates in urine. Ion exchange chromatography has been employed for the purification and group separation of urinary estrogen conjugates (Hahnel 1965; Musey *et al*. 1977; Setchell *et al*. 1976; Järvenpää *et al*. 1979) and the separation of free estrogens from neutral steroids (Eberlein 1969; Kaplan & Hreshchyshyn 1971; Kaplan & Hreshchyshyn 1972; Adessi *et al*.

1975). More recently, Fotsis (1987b; Fotsis & Adlercreutz 1987a) has discussed extensively the many problems in group separation of estrogen conjugates with ion exchange chromatography for complete estrogen conjugate profiles. New classes of diphenolic compounds, mammalian lignans and isoflavonoids, which are weak acids, have been detected for the first time in biological fluids due to their retention on the anion exchanger, thus interfering with GC analysis of steroids. The interference is most extensive in the region where the α-ketolic estrogens are eluted in GC and has in the past resulted in gross overestimation of some of these compounds (Bannwart *et al*. 1988a).

An important step forward in field of phytoestrogen analysis in biological material was the observation (Fotsis & Adlercreutz 1987) that phytoestrogens, the compounds with two phenolic hydroxyls, may be separated from estrogens on a QAE-Sephadex ion exchanger in the bicarbonate form. Both groups of compounds are retained on the resin in methanol; estrogens with one aromatic hydroxyl group can be eluted with 80% methanol and the diphenolic lignans and isoflavonoids with 0.1 M acetic acid in 80% methanol.

Two ion exchange procedures were finally adopted.

I. Chromatography on DEAE-Sephadex in the hydroxyl form (DEAE-OH^-).

The removal of neutral steroids was achieved using the free base form of DEAE-Sephadex. The gel was packed in methanol to a column dimension of 0.5 x 1.5 cm in a Pasteur pipette with a small piece of cotton in the bottom. The sample was applied in 0.5 ml of methanol, the column being thereafter eluted with the same solvent; this fraction, containing neutral steroids, was discarded. Diphenols were obtained by eluting with 5 ml of 0.1 M acetic acid in methanol. This fraction was evaporated to dryness, dissolved in 500 ml of methanol and then transferred to the next step.

II. Chromatography on QAE-Sephadex in the acetate form.

Remaining impurities (organic acids and a considerable amount of the chromogens) were removed by QAE-Sephadex in the acetate form. The exchanger was packed in methanol to give a column dimension of 0.5 x 3 cm in a Pasteur pipette. The sample was applied (in 0.5 ml of methanol) and the column eluted with 4 ml of methanol. The first fraction (=4.5 ml) contained anhydroSECO, SECO and MAT. In addition, the QAE-Sephadex column was eluted with 7 ml of 0.2 M acetic acid in methanol. This fraction comprised biochanin A, formononetin, daidzein, genistein and coumestrol.

1.6. Derivatization

The fractions were evaporated to dryness under nitrogen, and the trimethylsilyl (TMS) ether derivatives were formed by reaction with 100 µl of pyridine / hexamethyldisilazane / trimethylchlorosilane (9/3/1 by volume) and incubation for at least 30 min at room temperature or overnight. Calibration standard mixtures were derivatized in the same way. Deuterated TMS ether derivatives (d_9-TMS) of internal standards were formed under the same conditions utilizing hexamethyl-d_{18}-disilazane and trimethyl-d_9-chlorosilane. After incubation the solvent was evaporated to dryness and the residue dissolved in *n*-hexane containing approximately 3% (by volume) Silyl 8. Subsequently the *n*-hexane extract was transferred to a microvial.

1.7. GC-MS

In the early stages of this investigation (I, II, III) an HP 5995 quadrupole mass spectrometer coupled online to an HP 59970C MS Chem Station and equipped with a 0.2 mm x 12.5 m bonded

phase BP 1 vitreous silica column (SGE) directly connected to the ionization chamber was used. The internal diameter (I.D.) was 0.22 mm and the outer diameter (O.D.) was 0.33 mm, the phase layer being 0.25 μm. Helium was used as a carrier gas. Generally, the temperatures of the transfer line, ion source and analyzer are 310, 250 and 250 °C, respectively. The temperature of the oven was first kept 1 min at 100 °C and then increased by 30 °C /min (maximum speed) to 280 °C.

Later (IV, V, VI), a Fisons MD quadrupole mass spectrometer and a Fisons 8000 gas chromatograph connected to a PC computer (MassLab programme) and equipped with the similar BP 1 vitreous silica column directly connected to the ionization chamber were used. The I.D. and O.D. were the same as in the HP system. In this case the temperatures of the transfer line, ion source and analyzer (oven and ionization chamber) were 270, 200 and 230-280 °C (depending on the compounds analyzed), respectively. The temperature of the oven was first kept at 150 °C for 1 min and then increased by 40 °C /min to 260 °C (isoflavonoids) and 270°C (lignans). When these temperatures were reached, the oven was heated gradually 5°C /min to 270°C and 3°C /min to 280°C in the case of isoflavonoids and lignans, respectively.

1.8. GC analysis of samples with high concentrations of isoflavonoids and lignans

Capillary column gas chromatography was carried out using an HP 5890 Series II Gas Chromatograph combined with a HP 7673A Automatic Sampler and coupled with an HP data system consisting of an HP 7673A Controller, an HP 9114B Disk Station and an HP 3396A Integrator for processing of chromatographic data. The instrument was equipped with a flame ionization detector and operated with helium as the carrier gas.

For samples with high amounts of isoflavonoids and lignans the same steps of the method were applied but no deuterated internal standards were used. After silylation a sample (residue) was dissolved in *n*-hexane containing approximately 3.4% (by volume) cholestane as the internal standard and 3% (by volume) Silyl 8.

2. Analysis of lignans in the biological samples (V, VI)

Urinary and plasma samples (V) were analyzed using TR-FIA methods for ENL in plasma (Adlercreutz *et al*. 1997; Adlercreutz *et al*. 1998b; Adlercreutz *et al*. 1998a) and urine (Uehara *et al.* 1999). The analytical procedure is briefly as follows. After addition of the radioactive internal standard ^{3}H-oestradiol glucuronide (only for plasma), hydrolysis reagent (0.2 U/ml of *ß*-glucuronidase and 2 U/ml of sulfatase in 0.1 mol/l acetate buffer, pH 5.0) was added to the plasma or urine samples which were mixed and then incubated overnight at 37°C. For plasma samples diethyl ether was used to extract free (unconjugated) ENL after hydrolysis; for urine no extraction was used. Then the hydrolyzed and extracted plasma or urine samples in assay buffer (Tris-HCl buffer containing 0.5% BSA, pH 7.8) were pipetted into prewashed goat anti-rabbit IgG microstrips. Simultaneously antiserum (dilution 1:250,000) and europium-labelled ENL (dilution 1:400,000) in the assay buffer were added into the microstrips. After incubating and shaking the strips slowly on a DELFIA plate shaker at room temperature for 90 min., the strips were washed with a DELFIA plate washer. Subsequently DELFIA enhancement solution was added to each well and the strips were shaken slowly for an additional 5 min.

In order to study the intestinal metabolism of lignans (VI), the concentrations of plant and mammalian lignans in intestinal digesta sampled along the intestinal tract of pigs were determined

by ID-GC-MS. In this investigation the concentrations of plant and mammalian lignans were determined in digesta from the ileum, caecum, middle colon and in faeces from pigs fed breads based on whole rye grains or on three different rye grain milling fractions. Following the addition of stable deuterated internal standards for all compounds to correct the losses during all the purification steps, the intestinal samples (VI) were extracted and purified in several ion exchange chromatographic steps according to Adlercreutz et al. (1995b). Using this methodology, only unconjugated lignans SECO, MAT, END and ENL were extracted from the intestinal samples and, following derivatization, measured by ID-GC-MS-SIM. Ileal samples were additionally hydrolyzed with acid and analyzed with a combination of both the methods for food samples and faeces. A flow diagram of the method modified is shown in the flow diagram (Fig. 4). Measurements of the plant and mammalian lignans (total conjugates and unconjugated lignans) liberated by this combined methodology were finally performed using ID-GC-MS-SIM. Stable deuterated internal standards of all the compounds were used for the correction of losses during the procedure.

Using the chromic oxide indigestible marker added to the diets, it was possible to estimate the quantitative recoveries of lignans in the intestinal segments. Chromic oxide was analyzed by colorimetry using the method of Schürch et al. (1950).

3. Calculations (V, VI)

Fluorescence (V) was read on a DELFIA Victor multilabel counter. Results were calculated according to the formula:

(ENL in plasma) Final result = Concentration (read) x l/recovery x dilution factor.

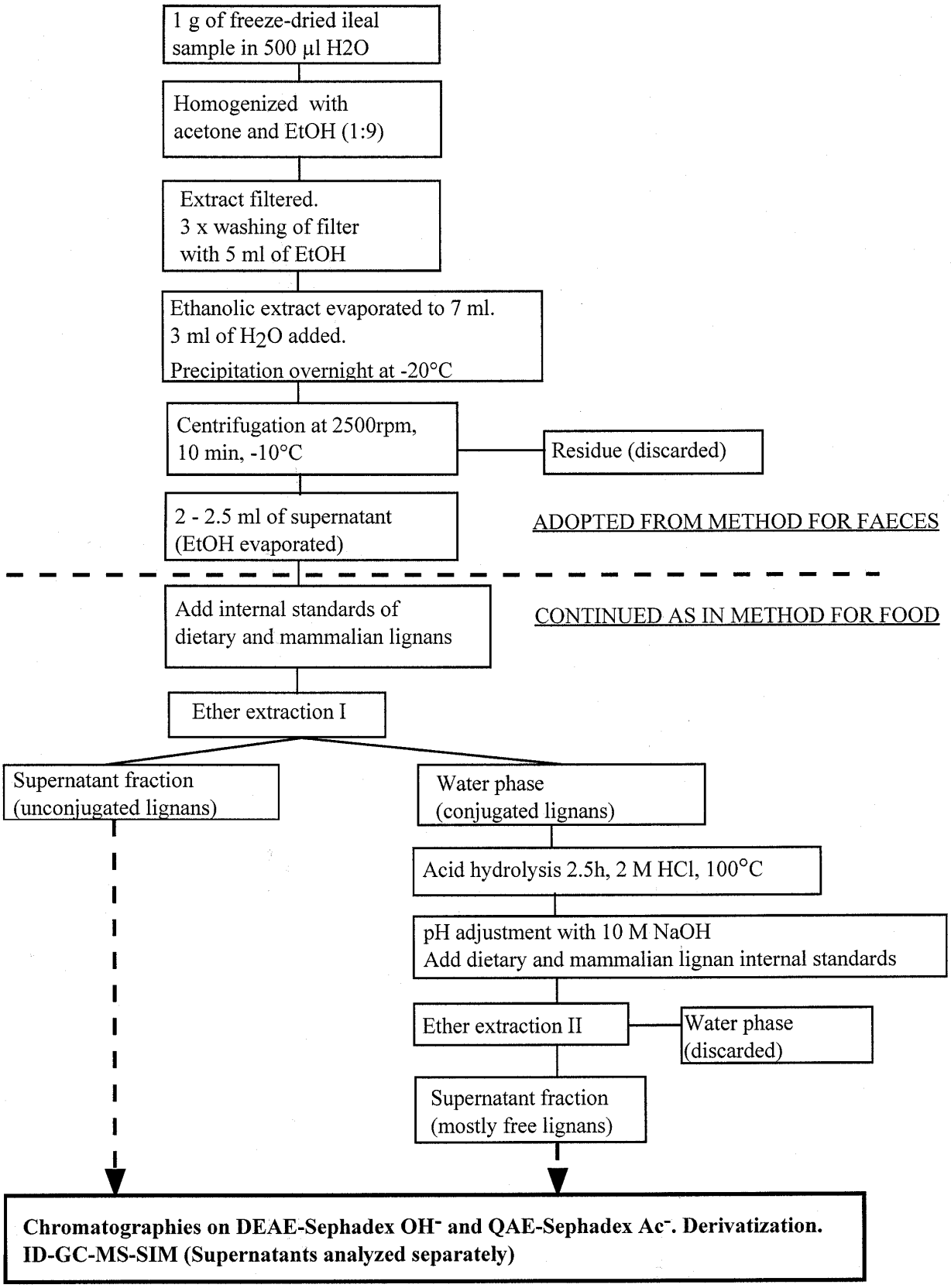


Fig. 4. Flow diagram of a combination of the methods for food samples and faeces. The method was applied to determine conjugated lignans in ileal samples (V).

(ENL in urine) Final result = Concentration (read) x dilution factor.

The theoretical content of lignans in the diets on the basis of the concentration of lignans in the rye raw materials ($Calc_{lignan}$) was calculated using arabinoxylans (AX) as in internal marker for the rye raw materials, and thus, assuming that the ratio of lignans to AX was the same in the rye raw materials and in the corresponding diets:

$$Calc_{lignan} = \frac{Lignan_{raw} * AX_{diet}}{AX_{raw}} ,$$

where $Lignan_{raw}$ and AX_{raw} are the concentrations of plant lignans and AX in the raw material, respectively, and AX_{diet} is the concentration of AX in the diet. In order to test this method of calculation, the theoretical content of starch from the rye raw materials to the rye diets ($Calc_{starch}$) was calculated using a similar formula:

$$Calc_{starch} = \frac{Starch_{raw} * AX_{diet}}{AX_{raw}} ,$$

where $Starch_{raw}$ is the concentration of starch in the raw material.

Recovery of the dietary plant lignans (R_{diet}; % of intake) as plant and mammalian lignans in the intestinal segments was calculated as shown in the following example for a caecum sample ($R_{diet(caecum)}$):

$$R_{diet(caecum)} = \frac{Lignan_{caecum} * Cr_2O_{3\,diet} * 100}{Lignan_{diet} * Cr_2O_{3\,caecum}},$$

where $Lignan_{caecum}$ is the concentration of plant and mammalian lignans in the caecum sample and $Lignan_{diet}$ is the concentration of plant lignans determined in the diet, and $Cr_2O_{3\,diet}$ and $Cr_2O_{3\,caecum}$ are the concentrations of chromic oxide in diet and caecum, respectively.

Similarly, the recovery of ileal lignans (R_{ileum}; % of ileal level) in the large intestine was calculated as shown in the following example for a caecum sample ($R_{ileum(caecum)}$):

$$R_{ileum(caecum)} = \frac{Lignan_{caecum} * Cr_2O_{3\,ileum} * 100}{Lignan_{ileum} * Cr_2O_{3\,caecum}},$$

where $Lignan_{ileum}$ and $Cr_2O_{3\,ileum}$ are the concentrations of lignans and Cr_2O_3 in the ileum, respectively.

The daily recovery of dietary lignans in the ileum ($R_{daily(ileum)}$; μmol/day) was calculated as:

$$R_{daily(ileum)} = \frac{Lignan_{daily\ intake} * R_{daily(ileum)}}{100},$$

where $Lignan_{daily\ intake}$ is the average daily intake of lignans at time of slaughtering.

The disappearance of lignans from the intestinal tract between the ileum and the caecum ($Dis_{ileum-caecum}$) was calculated as:

$$Dis_{ileum\text{-}caecum} = R_{daily(ileum)} - \frac{R_{daily(ileum)} * R_{ileum(caecum)}}{100}$$

4. Statistical methods

Values in the text (V, VI) were expressed as means ± SEM. The data were analyzed using one-way ANOVA (V, VI) and two-way ANOVA (VI). After one-way ANOVA, the data were assessed using Duncan's Multiple Range Test to determine whether mean values were significantly different. Differences were considered significant at P values < 0.05. Correlation coefficients (VI) for relationships between unconjugated lignans and conjugated lignans or recovery of dietary lignans in ileum were assessed using the least squares method (Pearson). Subsequently, the Pearson method was used to test correlation coefficients for plant and mammalian lignans, and recovery of dietary lignans or recovery of ileal lignans.

All the statistical analyses were performed with the SPSS statistical software package (Version 6.1J, SPSS, Chicago, IL, USA).

RESULTS

1. ID-GC-MS method for the determination of isoflavonoids, coumestrol and lignans in food samples

During the early stages of the present investigation an original method for identification and quantification of phytoestrogens was developed (I). The multistep procedure was carefully tested, optimized and validated. Each step of the method was described in detail and its relevance was discussed in the original publication. However, when the method was applied to analyze breads of a human rye/wheat bread experiment (Adlercreutz & Mazur 1997) and later in the pig study (V), a discrepancy between an intake of plant lignan precursors (SECO and MAT) in cereals and the urinary excretion of mammalian lignan ENL was observed. This observation posed a question whether the method underestimates the lignans in plant material and/or whether other precursors of mammalian lignans exist. In order to exclude a methodological error a number of experiments were carried out in order to test modifications of the original method (Fig. 5) and to improve analytical conditions and increase the yield of lignans released from plant matrix. Outcomes of the most important experiments are described below in this report. Table 4 compares the results, viz. the total concentrations of lignans SECO and MAT, between the experiments with a rye meal sample.

1.1. Results of studies on the hydrolysis of phytoestrogens

The hydrolytic removal of the carbohydrate component of isoflavone glycosides simplifies the quantitative analysis of the phytoestrogens. The principal target was to achieve complete hydrolysis of all glycosides of lignans and isoflavonoids without too much loss. The two first hydrolytic steps, viz. rehydration with distilled water and the *Helix pomatia* enzymatic hydrolysis, were very effective in hydrolyzing the isoflavonoid glycosides. Soaking the samples with H_2O might have activated plant endogenous enzymes and/or initiated weak hydrolytic

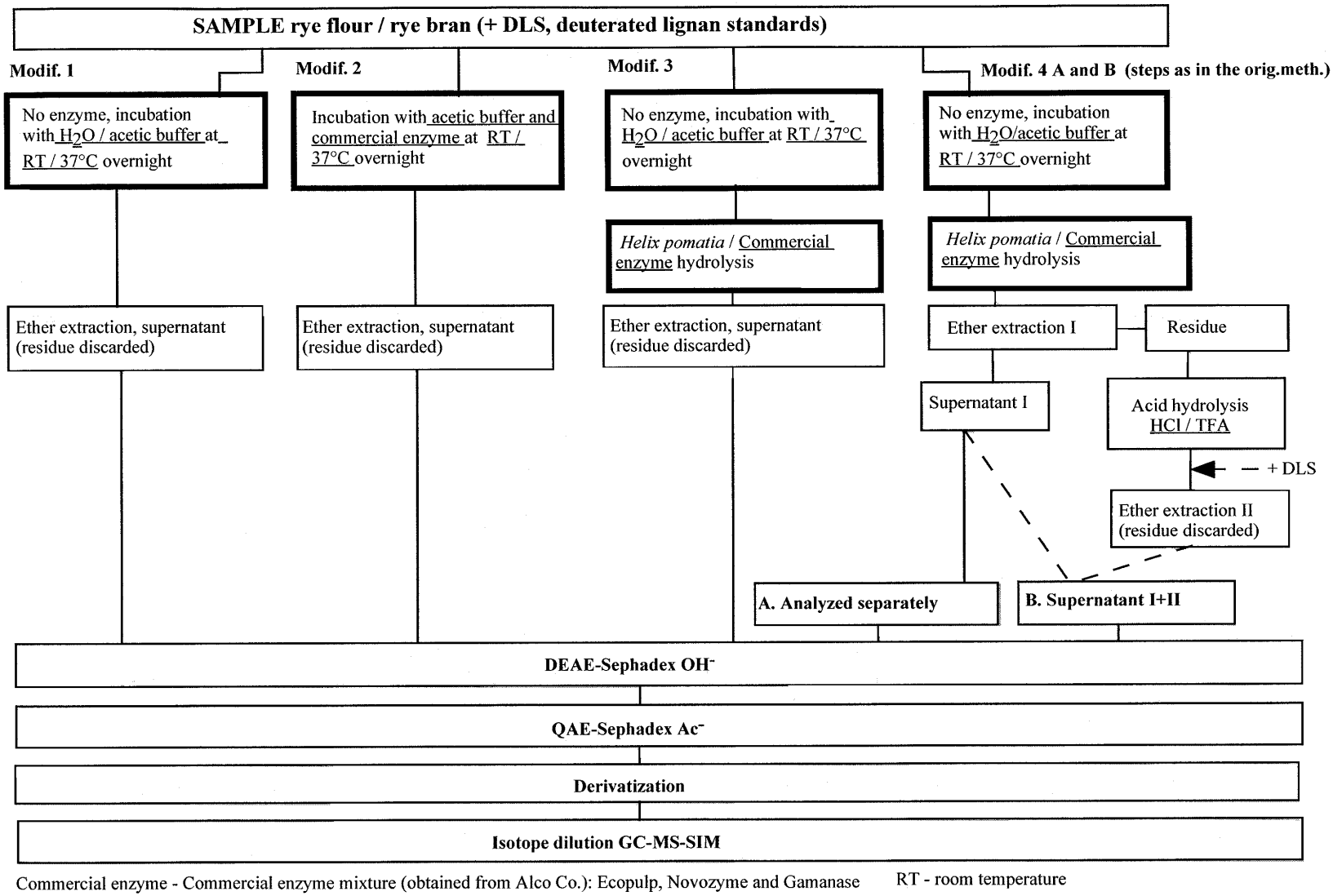


Fig. 5. Flow diagrams of modifications of the original method investigated in a number of experiments. The bold frames mark critical steps of the method changed and tested.

Table 4. Lignan concentrations in a rye meal sample analyzed in several experiments using I - IV modifications of the original method (Fig. 5). Results, expressed in μg/100g, are mean values of duplicates.

Modification	**Characteristics:**				**Total lignans**[1]	**Remarks**
	Sample	Incubation/temperature	Enzyme hydrolysis	Acid hydrolysis		
I	Rye flour	H_2O/RT	-	-	18.8	Hydrolysis by release
I	Rye flour	H_2O/37°C	-	-	24.5	endogenous plant enzy
II	Rye flour	Acetic buffer and commercial enzymes[2]/37°C		-	14.21	Enzymes not at optimu activity
III	Rye flour	H_2O/RT	Commercial enzymes[2]	-	11.1	
IV B	Rye flour	H_2O/RT	Commercial enzymes[2]	HCl	61.4	
III	Rye flour	H_2O/RT	*Helix pomatia*	-	27.5	
III	Rye flour	H_2O/37°C	*Helix pomatia*	-	30.9	
III	Rye flour	H_2O/37°C	Xylanase	-	35.2	
IV A	Rye flour	H_2O/RT	*Helix pomatia*	HCl	75.5	After E. hydrol. 27.6 After acid hydrol. 47.9
IV A	Rye flour	H_2O/37°C	*Helix pomatia*	HCl	81.8	After E. hydrol. 37.7 After acid hydrol. 44.1
IV B	Rye flour	H_2O/RT	-	HCl	71.5	
IV B	Rye flour	H_2O/RT	*Helix pomatia*	HCl	82.8	
IV B	Rye flour	H_2O/RT	Xylanase	HCl	90.4	
IV B	Rye flour	H_2O/RT	*Helix pomatia*	$C_2HF_3O_2$	85.5	Acid hydrol. at 120°C

[1] Anhydrosecoisolariciresinol, secoisolariciresinol and matairesinol

[2] Commercial enzyme mixture (obtained from Alco Co.): Ecopulp, Novozyme and Gamanase

RT – room temperature

activity of bacterial enzymes. The *Helix pomatia* enzyme reagent, however, was insufficient to hydrolyze complex lignan compounds from cereal samples or did it at low rate. A collaborative study with Nilsson (1997a) showed that lignans in rye are localized in the outer fibre-containing layers, with the highest concentration in the aleurone and pericarp/testa layers (Nilsson *et al*. 1997a) containing phytin, polyphenols, enzyme inhibitors and other compounds generally regarded as antinutritional factors. The 1 to 3 cell thick aleurone layer is tightly bound to the outer fibre layers, and the liberation of the lignan precursors from these very resistant cell wall cells is difficult. In order to investigate whether other enzymes could be more active and able to increase the recovery of dietary lignans, such enzymes as cellulase, xylanase, pectinase, mannanase, protease and amylase were tested in experiments carried out in collaboration with H. Härkönen at VTT, Espoo, Finland. Xylanase yielded a higher concentration of lignans compared to the other enzymes (*Helix pomatia* was not included in that specific assay; data not shown). This observation led to a series of experiments on the hydrolysis of rye flour or rye bran treated with xylanase. Rehydration with H_2O overnight at room temperature and incubation at 37°C was also tested. The activity of xylanase in the enzymatic hydrolysis turned out to be as high as that of *Helix pomatia*, or in some cases slightly higher. This means that the treatment of samples with xylanase may yield a slightly improved recovery, but the difference was within the analytical error of the method and, owing to the small number of samples (n = 2), not statistically significant. Generally, however, no enzyme treatment alone was sufficient to hydrolyze bonds in lignan glycosides or to liberate the glycosides from more complex structures in the cell wall. The next step after diethyl ether extraction, HCl hydrolysis, was indispensable to achieve the highest recovery of lignan aglycones.

Lignan glycosides in grain and cereal samples proved to be very difficult to hydrolyze. Simultaneously, the lignan aglycones proved to be sensitive to heat and acid treatment. If SECO standard was subjected to acid hydrolysis, a significant destruction of SECO was observed. This was not the case for isoflavones, which were surprisingly stable during heat and acid treatment. In paper V, all raw material and dietary samples were additionally hydrolyzed with TFA acid (instead of HCl) in a parallel experiment in order to control for results. Concentrations of lignans obtained using this modification were the same or slightly higher than the results of the standard method. The differences, however, were not statistically significant.

Most recently, NaOH hydrolysis was extensively tested with regard to release of lignans from cereal samples (Fig. 6). The results of several experiments (different base molarity, different duration of hydrolysis, different temperature) showed that the NaOH treatment, when included as an additional step in the method, did not increase the yield of lignans SECO and MAT from rye flour and rye bread samples.

1.2. Extraction

One method to extract free isoflavones and lignans (aglycones) from an aqueous solution is to partition it with an organic solvent immiscible in water; however this method requires the compounds to be soluble in the solvent. When aqueous methanol, acetonitrile and diethyl ether were tested as extraction solvents, recovery of isoflavones after extraction with acetonitrile was poor. Although aqueous methanol recovery was similar to that of diethyl ether, in consideration of long experience in this laboratory and for practical reasons (e.g., fast and easy evaporation), diethyl ether was chosen for the extraction of phytoestrogen aglycones after the enzymatic and

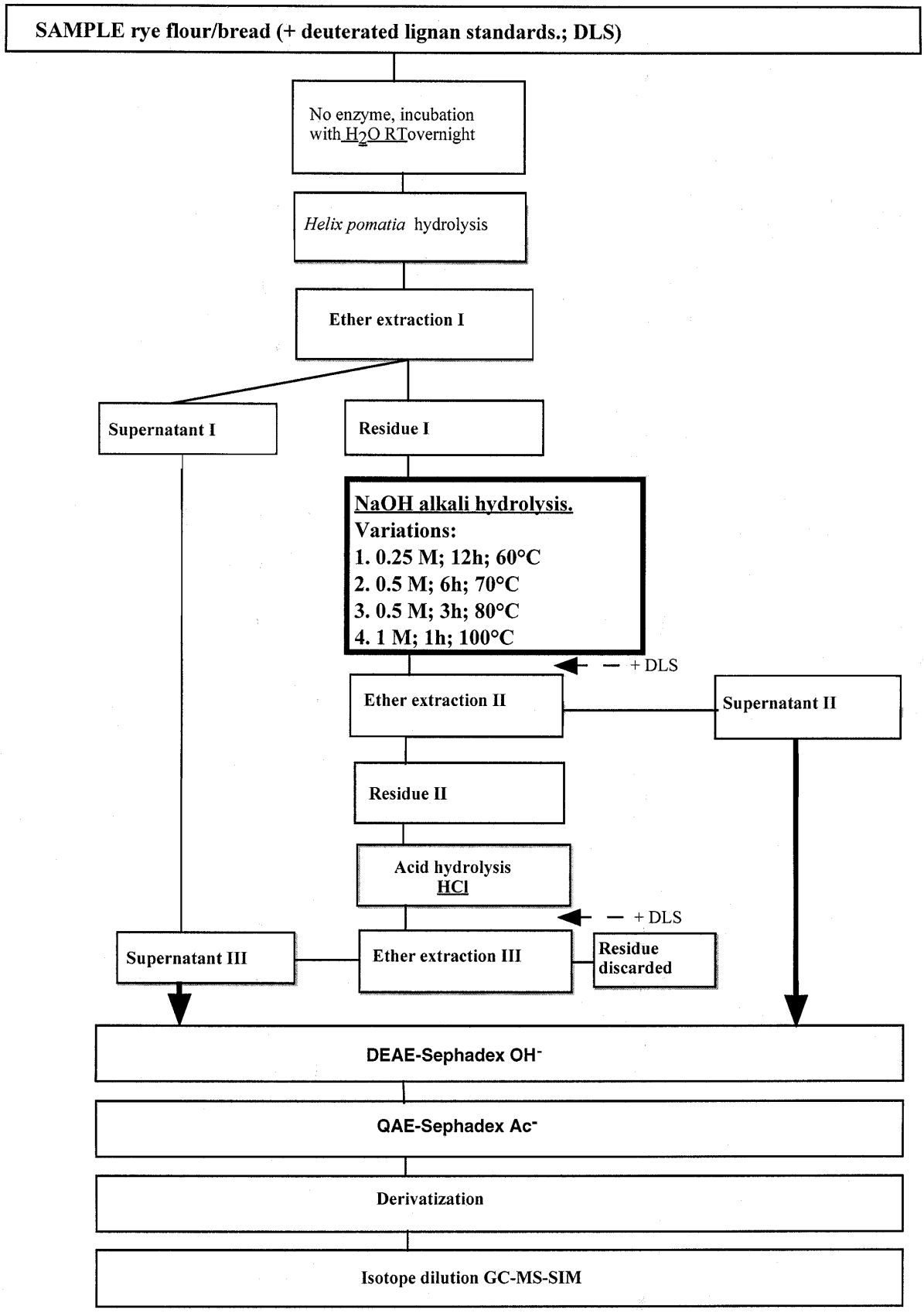


Fig. 6. Flow diagrams of the original method modified to include NaOH hydrolysis

acidic hydrolyses. Diethyl ether was also found to yield the best results with regard to degree of purification of the sample. An experiment was performed in order to check whether the ether extraction is sufficient to extract all free isoflavonoids and lignans and whether no phytoestrogens are discarded. When the water phase, viz. the waste fraction after the ether extraction II, was analyzed for phytoestrogen content only trace amounts, if any, were found (T. Nurmi, personal communication) when a very sensitive HPLC method with coulometric electrode array detector was applied to the samples (Nurmi & Adlercreutz 1999b).

2. Validation of the method

2.1. Recoveries and accuracy

The accuracy of the assay was determined in two types of experiments. First, authentic reference standards were added to water (n=20) and processed through the whole procedure. In other experiments (n=10) standards were added to a meal with relatively low phytoestrogen concentration. The concentration levels of the added synthetic standards were accommodated in the middle of the standard curve in GC-MS to give highest precision. Mean deuterated internal standard corrected recovery for all the analyzed phytoestrogens was close to 100% (MV 99.5 % ± 7.9) in both experiments.

2.2. Imprecision

When analyses were carried out with this method, duplicate control samples I and II (consisting both of the same components but mixed in slightly different proportions) with known amounts of isoflavonoids and lignans (except coumestrol) were included in all series of analyses. The means

of the duplicates were used when calculating method imprecision. The inter-assay coefficient of variation (CV) of the control samples I (n=9) and II (n=14) was found to range from 4.5% to 14.6% (average CV 10.0%), and from 7.0% to 21.2% (average CV 12.5%), respectively. The control sample II used for the studies of method inter-assay imprecision had a very low concentration of biochanin A (5.2 μg/100g; close to the sensitivity limit), resulting in a CV of 21.2 %. However, the other results were found to be satisfactory. The intra-assay CVs calculated from the control sample II (n=13) ranged from 3.1 to 9.6 (average CV 5.9%).

2.3. Specificity

The specificity of the method is high as a result of the quantification mode used, viz. selected ion monitoring (SIM) combined with effective separation using capillary columns and deuterated internal standards. The specificity may be manually checked by computer for every peak if there is some doubt about it and because the use of deuterated internal standards allows efficient utilization of the retention times as a means of checking the specificity.

2.4. Sensitivity. The lowest detectable amount of phytoestrogen, the sensitivity limit of the method, defined as two to three times the standard deviation of the assays at low levels, was approximately 2-3 μg/100g.

3. Results of quantitative assays

3.1. Phytoestrogens in foods and diets (I, II, III, IV, V, VI)

The method - ID-GC-MS in the selected ion monitoring mode - was successfully applied for the quantitative determination of the 4 isoflavones formononetin, biochanin A, daidzein, genistein, and coumestrol, and simultaneously the 2 lignans SECO and MAT, in plant-derived foods. These phytoestrogens were quantified using synthesized deuterated internal standards for the correction of losses during the procedure. Because of the conversion of SECO to anhydroSECO during acid hydrolysis, the method was further developed to include also this lignan (not occurring in nature). The sum of SECO and anhydroSECO was used as an estimate for the presence of SECO in the food samples.

The isoflavones enjoy widespread distribution in most of the members of the Leguminosae family, including such prominent high-content representatives as soybean, clover, mung bean, alfalfa, peanut and kudzu (*Pueraria lobata*) (II). The highest total concentration of isoflavones, with regard to edible seeds, was found in kudzu root (over 200,000 μg/100g), followed by soybeans (ranging from 37,300 μg/100g to 140,300 μg/100g) and chickpea (1,150-3,600 μg/100g). All the legumes, except the "Green split" pea and 'Canada' faba bean, contained 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 or trace concentrations (up to 10 μg/100g). The legumes soybean, kudzu leaf, and red clover contained more (105.0-1,570 μg/100g). The richest source of coumestrol in human food that we have found is mung bean sprouts, which contain 20 times as much coumestrol (about 1,000 μg/100 g) as do alfalfa sprouts (45 μg/100g). SECO at the concentration of 1,590 μg/100g was detected in *Sophora japonica*. However, of the most commonly known and consumed legume species analyzed, the soyabean and the peanut (*Arachis hypogaea*) contained the highest level of SECO (13.3-273.0 μg/100g and 333.0 μg/100g, respectively), but no or minor amounts of MAT

could be detected. Generally, all the other legume items analyzed contained SECO (from 2.8 to 475.8 μg/100g). Red clover, a cause of "clover disease" in sheep in Australia, was found to contain the highest concentrations of formononetin and biochanin A.

Linseed is the most abundant source of lignans in foods, the main component being SECO with minor amounts of MAT (I). When linseed is cracked and defatted, SECO content rises to 600,000 - 700,000 μg/100g dw, which yields by far the highest concentration of lignans from plant sources. In other oilseeds such as sesame, clover, sunflower, caraway and poppy seeds, significantly lower concentrations of lignans were found (SECO 13 - 610 μg/100g, MAT 0 - 608 μg/100g dw; IV). Together with SECO and MAT in all oilseeds (except linseed) we determined daidzein and genistein. Nuts (peanut, cashew, hazelnut, pistachio, walnut and almonds), containing substantial amounts of SECO (ranging 96 - 257 μg/100g dw) are poor sources of isoflavones and MAT (IV). Oils derived from oilseeds (including soy oil) contain only trace amounts of phytoestrogens.

Cruciferous vegetables contain small amounts of daidzein and genistein ranging from 0 to 6 μg/100g and 5 to 14 μg/100g dw of daidzein and genistein, respectively. SECO concentrations, however, were higher and varied from 33 μg/100g in cabbage to 414 μg/100g dw in broccoli. MAT was detected in broccoli, whereas in the other cruciferous plants analyzed it was found in trace amounts. Onion, garlic and chives (allium vegetables) were found to contain relatively high concentrations of SECO (83, 380 and 1,254 μg/100g dw, respectively) but no isoflavones. In general root, orange and green leafy vegetables do not contain isoflavones. However, concentrations of SECO vary from 10 μg/100g dw in potato and 114.4 μg/100g dw in celery to 817 μg/100g dw, 3,070 μg/100g dw and 3,870 μg/100g dw in zucchini, asparagus and pumpkin

(peeled), respectively, with average amounts exceeding 100 μg/100g dw. MAT, which increases in plants under fungal attack, has been found only in minimal amounts.

Surprisingly no isoflavones could be measured in such fresh fruits as tomatoes (sometimes considered a vegetable), apples and pears, stone fruits, citrus fruits and tropical fruits (bananas, melons, dates, pineapple and exotic species such as litchi, mangoes and guavas); however, the lignan SECO occurred in different amounts in all fruits. Common apple, banana and plum contain low, or even trace, concentrations of SECO. On the other hand, such exotic fruits as otaheite gooseberry (*Phyllanthus acidus*) or pomegranate (*Punica granatum*) concentrate much higher amounts of this lignan (3,040 and 814.6 μg/100g dw, respectively). SECO content of citrus fruits (lemon, orange, grapefruit), kiwi fruits and melons, nowadays readily available from greengrocers, varies from 41.9 to 1,164 μg/100g.

The analysis of green and black teas (I) and white and red wines yielded relatively high levels of the lignans SECO (teas: 561-2,890 μg/100 g) and MAT (teas: 56-413 μg/100 g) but only low levels of isoflavonoids. Coffee meal samples processed according to the original method were shown to contain 2-3 times less lignans SECO (393-716 μg/100g) and no MAT. Cocoa, called the 'food of the gods' due to its status in the ancient cultures of Central America, contained the lowest amounts of SECO, 44 μg/100g, and, similarly to teas and coffees, no MAT and just traces of isoflavones.

None of the isoflavonoids was measurable with our technique in eight berry samples (IV), mainly due to their low concentrations and some interfering compounds at these very low (trace) levels. On the other hand, relatively high levels of the lignan SECO were found in all the berries, ranging from 1,390 μg/100g (raspberry) to 3,718 μg/100g (bramble) dw. Bramble contained the

highest concentrations of SECO and total plant lignans. Strawberry, the most common Finnish berry, contained 1,505 μg/100g dw.

Table 5 shows the concentration of isoflavonoids and lignans in the food items analyzed.

A great deal of work has been done on the analysis of cereal raw materials and cereal products within the framework of the NordFood Industry project. Frequently the terminology used to characterize these staple foods is misapplied and could be misleading. Particularly two terms, 'whole grain' and 'whole meal', require an explanation. In this study the 'whole grain' was used to describe a final bread product containing all fractions of a grain (i.e. endosperm, aleurone, pericarp-testa and a germ) occurring in their natural proportions. The term 'whole meal' applies to a food that is a product made from a mixture of fibre and meal fractions mixed after fractionation in various proportions which must be known. The germ is also in the whole grain unless it is removed. Rye meal, rye grain milling fraction (Nilsson *et al*. 1997a; 1997b) and rye bread samples, and other cereals such as wheat, rice, maize, barley, oats, and their products (Adlercreutz & Mazur 1997) were analyzed and found to contain substantial amounts of the lignans SECO and MAT (range 8-132 μg/100g and 0-167 μg/100g dw, respectively), but no or trace concentrations of isoflavones. Using ID-GC-MS-SIM techniques we have observed that the lignans are localized in the outer fibre-containing layers, with the highest concentration in the aleurone and pericarp/testa layers (Adlercreutz 1990; Nilsson *et al*. 1997a) containing phytin, polyphenols, enzyme inhibitors and other compounds generally regarded as antinutritional factors. The 1 to 3 cell thick aleurone layer is tightly bound to the outer fibre layers, and the liberation of the lignan precursors from these very resistant cells is difficult (I). Because of its close association with the outer fibre layer, modern milling techniques usually eliminate the

Table 5. Phytoestrogen content of various foods analyzed using the ID-GC-MS-SIM method (μg/100 g dw)

Plant foods Trivial name **(***Botanical name)*	**DAIDZEIN**	**GENISTEIN**	**SECOISOLARI-CIRESINOL tot**[+]	**MATAIRESINOL**	**SOURCE**
Grains and cereals					
Rye (*Secale cereale*), whole meal	0	0	47.1	65.0	(Adlercreutz & Mazur 19
Wheat (*Triticum dicoccum*), white meal	tr	tr	8.1	0	(Adlercreutz & Mazur 19
Barley (*Hordeum spp*), whole meal	14.0	7.7	58.0	0	(Adlercreutz & Mazur 19
Oats (*Avena sativa*), white meal	0	0	13.4	tr	(Adlercreutz & Mazur 19
Oilseeds and nuts					
Flaxseed (*Linum usitatissimum*)	0	0	369,900	1,0871	I
Sunflower seed (*Helianthus*)	8.0	13.9	610	0	I
Clover seed (*Trifolium spp*)*	178.0	323.0	13.2	tr	III
Walnut (*Juglans nigra*)	5.0	tr	163.0	5.0	IV
Hazelnut (*Corylus avellana pontica*)	tr	tr	119.0	4.0	IV
Fruits					
Apple (*Pyrus malus*)	12.4	tr	tr	0	IV
Plum (*Prunus domestica*)	0	0	5.0	0	IV
Banana (*Musa sapientum*)	0	0	10.0	0	IV
Papaya (*Carica papaya*)	0	0	8.2	0	(Mazur 1998)
Guava (*Psidium guajava*)	0	0	699.7	tr	(Mazur 1998)

Cruciferous vegetables					
Cabbage (*Brassica oleracea*)	tr	tr	33.0	tr	IV
Broccoli (*Brassica oleracea italica*)	6,0	8.0	414.0	23.0	(Adlercreutz & Mazur 19
Cauliflower (*Brassica oleracea botrytis*)	5.0	9.0	97.0	tr	IV
Allium vegetables					
Onion (*Allium cepa*)	0	0	83.0	8.0	IV
Garlic (*Allium sativum*)	tr	tr	379.0	3.6	(Adlercreutz & Mazur 19
Other vegetables					
Potato (*Ipomea batatas*)	0	0	10.0	6.0	IV
Carrot (*Daucus sativus*)	tr	tr	192.0	3.0	(Adlercreutz & Mazur 19
Pepper (*Capsicum species*)	0	0	117.0	7	IV
Beverages, wines (origin)**					
Chardonnay (Italy), white	nd	nd	135.5	17.2	(Mazur 1998)
Cabernet Sauvignon (France), red	nd	nd	686.0	74.1	(Mazur 1998)

[+] - Sum of anhydroSECO and SECO

tr - Present in trace amounts

nd - Not determined due to low concentrations and interferences by other compounds.

* Additionally, contained formononetin 1,270, biochanin A 381.0, and coumestrol 5.0.

** Given for volume, µg/l

aleurone with the pericarp/testa layers, which are therefore seldom present in commercial products. This is so particularly in Western societies.

Raw materials and dietary samples of the pig study (V) were analyzed using the standard method and the modified method (TFA acid hydrolysis). In the raw materials, plant lignan content varied from 45.8 μg/100g (1.3 nmol/g; whole rye) to 178.5 μg/100g (5.0 nmol/g; pericarp/testa) and in the diets - from 84.1 μg/100g (2.4 nmol/g; whole rye and endosperm) to 356.8 μg/100g (10.3 nmol/g; pericarp/testa).

3.2. Concentrations of ENL in plasma and urine of subjects fed a strawberry meal (V)

Basal and post-challenge ENL concentrations in plasma and urine varied greatly between the subjects (plasma: 1.7-22.4 nmol/l and 0.9-30.2 nmol/l; urine: 303.6-3128.4 and 99.8-7,071 nmol/l, respectively). The recovery of plant lignans SECO and MAT determined as urinary ENL (nmol/24h) varied from -23 to 438 % (mean 114%) for the 7 subjects.

3.3. Quantification of lignans in intestinal samples and faeces from the pigs (VI).

The concentration of total lignans (plant and mammalian), irrespective of diet, was highest in the ileal content samples, and the majority of the lignans were conjugated at this site. More than 95% of the conjugated lignans in the ileum were plant lignans, while the mammalian lignans constituted higher percentages of the unconjugated lignans (21-42%).

In the samples from the large intestine the concentration of plant lignans decreased markedly from the ileum to the caecum, whereas the concentration of mammalian lignans increased in the caecum samples and further in the middle colon and faeces for the pigs fed the

pericarp-testa or the aleurone diets, respectively. The faecal concentration of mammalian lignans was thus significantly higher for the pigs fed the pericarp-testa or the aleurone diets compared to that found in other pigs.

The ileal recovery of dietary lignans ranged from 251% (in pigs fed the endosperm diet) to 1,052% (in the pigs fed the whole rye diet). Approximately half of the ingested endosperm lignans were recovered in faeces, whereas 2-3 times as much lignans as had been ingested were recovered in the faeces of the pigs fed the three other diets (i.e. recoveries ranged from 216 to 360%). In the caecum, the recovery of ileal lignans ranged from 21% in the pericarp-testa sample to 42% in the whole rye sample, which may indicate that the majority of ileal lignans disappeared from the lumen before they arrived to the caecum.

DISCUSSION

1. Food Method

The ID-GC-MS-SIM method allows a specific, quantitative, reproducible, and sensitive determination of all the 7 most studied phytoestrogens in food samples, viz. daidzein, genistein, biochanin A, formononetin, coumestrol, matairesinol and secoisolariciresinol. Although many methods for the separation and quantification of phytoestrogens in plant (food) extracts by HPLC have been described (Wang *et al*. 1990; Eldridge 1982b; Coward *et al*. 1993; Murphy & Wang 1993; Jones *et al*. 1989; Franke *et al*. 1994; 1995; 1998; Fakutake *et al*. 1996; Saloniemi *et al*. 1995; Dorr & Guest 1987; Petterson & Kiessling 1984; Song *et al*. 1998; Murphy *et al*. 1982; 1999), some lack analytical data validating the method. They have also not been applied to a sufficiently representative number or range of food samples to establish method suitability and

robustness for routine use. Many failed to include some of the major phytoestrogens of biological interest. In order to ensure good quality and to control for losses and degradation of the compounds throughout a multistep method, internal standards should be used for all the compounds measured. An ideal internal standard should be a compound structurally related to the analyte and having a similar polarity but with a retention time that does not overlap with the other peaks in the chromatogram. Only few isoflavone investigators have used internal standards to adjust for analyte loss in extraction and analysis (Jones *et al*. 1989; Eldrige, 1982a Coward *et al*. 1993; Wang *et al*. 1990; Franke *et al*. 1994; 1995; Song *et al*. 1998; Murphy *et al*. 1999). Most compounds used as internal standards are either not structurally related to the analytes or have retention times that interfere with the isoflavone peaks. Precision and accuracy decrease considerably when only one internal standard to control for losses of more than one compound is used. It has to be kept in mind that different phytoestrogens bind differently to the food matrix. The ID-GC-MS-SIM method utilizes deuterated internal standards for each isoflavonoid and lignan by exchanging hydrogen atoms for deuterium atoms. Consequently, the retention times of the nondeuterated compounds and the deuterated standards are very close. The main drawback of this method is that it is relatively complicated mainly because so many compounds are assayed and because high sensitivity is needed.

Specificity and especially sensitivity of earlier methods are not sufficient for food samples with low concentrations of the compounds, and isoflavonoids and lignans were never measured in the same assay. Particularly at low levels HPLC with DAD and UV detectors, being photochemical detection systems, may easily give rise to non-specific results. HPLC method detection limits, obtained from authentic standards, are from two to hundred times higher than

those determined by GC-MS-SIM (Willard *et al*. 1988). Most of the HPLC methods have been developed using relatively rich sources of isoflavones, such as soybeans, soy products or synthetic isoflavones, and they report data for the richest sources. There is little or no quantitative information on foods with low, but not zero, phytoestrogen content, and these may be commonly present in Western-style diets. Despite the obvious need to validate a method over a wide concentration range much lower than that in soy-based products, this has not been done in most cases. Moreover, isoflavonoid content of foods (chiefly soy-derived) is inconsistent and varies from study to study, from laboratory to laboratory and from species to species. However, when we compared our results for daidzein and genistein in soybeans with those of some other reports (Franke *et al*. 1995; Petterson & Kiessling 1984; Wang *et al*. 1990) the concentrations are similar.

In three studies (Obermeyer *et al*. 1995; Rickard *et al*. 1996; Thompson *et al*. 1997) separate measurements of lignans in linseed have been reported. The amounts of SECO have been 4-5 times lower in these studies (Obermeyer *et al*. 1995; Rickard *et al*. 1996; Thompson *et al*. 1997) compared to those obtained in this laboratory (I, IV). Significant differences in linseed lignan content have been observed among varieties (Thompson *et al*. 1997) and for soybean isoflavones (Eldridge & Kwolek 1983). However, in this case it is more likely that other factors, probably methodological, are responsible for this five-fold difference. We can measure also the dehydrated anhydroSECO product of SECO, which has not been done with other methods. Furthermore, our harsh acid hydrolysis conditions (2M HCl, 100°C, 2.5 h), the step after enzymatic hydrolysis, are more effective in liberating the lignan aglycones from their glycosidic forms than is enzymatic treatment with ß-glucuronidase (Obermeyer *et al*. 1995) or *in vitro*

fermentation with human faecal inoculum (Thompson *et al*. 1997). The substantial amounts of MAT measured in linseed are probably a result of the more sensitive and very specific GC-MS detection system compared with gas chromatography and HPLC. The excellent sensitivity and selectivity, coupled with the small sample amount required (5-50 mg) for this assay, render it useful for the analysis of solid biological samples, such as tissue or intestinal samples (V), in experiments requiring study of both precursors and end products of metabolism. On the other hand, the small sample weight requires high sample homogeneity, which is sometimes difficult to achieve.

An unresolved problem is the underestimation of plant lignans in various rye raw material and rye diets produced with these raw materials (VI). The largest difference between raw material lignan contribution and the actual concentration in a diet was observed for the pericarp-testa diet, where the lignan concentration was 9 times higher than the calculated raw material contribution. It is obvious that the lignan content of cereals may also be underestimated (IV) (Adlercreutz *et al*. 1999a). With regard to the determination of lignans in foods, each step of the GC-MS method, for example such as the enzyme hydrolysis, acid hydrolysis and diethyl ether extraction, has been carefully tested and found to be methodologically valid. When validating the method, however, we found 15-20% destruction of free anhydroSECO and SECO standards during acid hydrolysis. This observation could imply lignan underestimation in the range of 15-20% due to rigorous acid/temperature treatment. Nevertheless, in the case of complex matrix samples, i.e., containing lignans in their natural form as glucosides, the following must be kept in mind: 1) theoretically at the beginning of the acid hydrolysis there are no free lignans (extracted with diethyl ether in a preceding step); 2) the presence of other organic material may prevent or slow down the

destruction; 3) when resistant SECO glycosides are hydrolyzed with acid, the free compounds are converted to anhydroSECO, which is also determined. Moreover, in this laboratory some cereal samples were recently analyzed independently with a HPLC method, which is a modification of the GC-MS method. The major difference is the use of an HPLC instrument with a coulometric electrode array detection system that is approximately 1,000 times more sensitive than the commonly employed UV or diode array detectors. Generally the GC-MS and HPLC results for plant lignans were similar; differences between the concentrations of SECO and MAT quantified using both methods were not statistically significant.

However, one explanation could be that the cell wall structure and linkages between components of the cell wall and the lignans are not broken during hydrolysis, thus accounting for the underestimation of lignans in the cereals, particularly in the high-fibre rye raw materials. Similarly to other cell wall esterified phenolic compounds (Eraso & Hartley 1990), dimers, trimers and higher oligomers of the lignans may occur (Ayres & Loike 1990) and these probably contain ether bonds that are not broken in the method used (see also 4.3). However, results of determination of high concentrations of SECO in other foods (e.g. linseed and legume seeds) and in beverages suggest that the method is efficient in quantification of lignans. Processing of food (e.g. making bread) involving bacterial or yeast fermentation and high temperatures could change characteristics of plant cell structure and may consequently relate to an increased availability of the plant lignans. The effect of baking bread on the availability of plant lignans in raw material, dough and the bread was investigated. Results, however, showed that no increase in concentration of lignans was observed in processed whole grain rye flour (Härkönen *et al*. 1999).

Concentrations of isoflavonoids in soy-derived foods are in agreement with those published in the literature, and their levels, when tested in a recent metabolic study in man (Watanabe *et al*. 1998), did not indicate a discrepancy between the dietary intake and the urinary and faecal excretion. In this study seven men consumed 60 g of Kinako (baked soybean powder containing 103 μmol daidzein and 112 μmol genistein). The total recovery of daidzein, *O*-DMA and equol from urine and faeces was 54.7%, calculated from daidzein intake, while 20.1% of administered genistein was recovered as genistein. High individual variations between the subjects were reported.

2. Quantitative assays in various foods and beverages

When the GC-MS method had been developed the analysis of popular staple foods in various populations was initiated in order to find out whether a correlation could be found between the consumption of specific foods or beverages and decreased incidence of chronic diseases, particularly in Asian populations. The previous detection of high concentrations of biologically active isoflavonoids and mammalian lignans in urine and plasma of subjects living in areas with low cancer and CHD incidence suggested that isoflavones and lignans derived from these foods may prevent chronic diseases like cancer and CHD (Adlercreutz 1990). The everyday consumption of legumes (mainly soy in different forms), teas (particularly green tea), and rice differentiate the oriental diet from Western cuisine. Moreover, the beneficial properties of these foods with regard to cancer and cholesterol metabolism had been acknowledged before the phytoestrogens were identified, and they were utilized in popular medicine to treat a variety of diseases (Potter & Steinmetz 1996).

The members of the Leguminosae family have been shown to contain, apart from isoflavones, a spectrum of chemicals (Slavin 1991; Messina & Barnes 1991) such as protease inhibitors, saponins, phytosterols, and inositol hexaphosphatase, each of which has been shown to inhibit carcinogenesis in animals or *in vitro*. Legume seeds are rich in complex carbohydrates (both starch and dietary fibre) which have been reported to be inversely associated with cancer and other pathological conditions of the colon (Kritchevsky 1986). In order to test our assumption concerning the origin of phytoestrogens in the biological fluids of oriental populations with low breast and prostate cancer risk, the GC-MS method was applied to analyse of a number of Leguminosae samples. The plant lignans SECO and MAT were measured in 68 edible legumes (III, IV) for the first time. The concentrations of phytoestrogens in legumes seem to be in good agreement with results of studies relating urinary phytoestrogen excretion to diet (Adlercreutz *et al*. 1986a; 1991a; 1994; Hutchins *et al*. 1995a; Lampe *et al*. 1995). Interestingly, daidzein (and genistein) extracted from kudzu root (*Pueraria lobata* or *Radix puerariae)*, containing more daidzein than soy, has been reported to inhibit human alcohol dehydrogenase (ADH) isoenzymes. This property explains the biochemical basis for the traditional use of *Radix puerariae* in the treatment of alcohol-related diseases in China since ancient times (Keung & Vallee 1993a; Keung 1993). Red clover, a cause of "clover disease" in sheep in Australia, was found to contain the highest concentrations of formononetin and biochanin A.

Consumption of tea (*Camellia sinensis*) has been advocated to play a role in prevention of cancer, coronary heart disease and atherosclerosis (Young *et al*. 1967; Imai & Nakachi 1995; Kohlmeier *et al*. 1997; Jankun *et al*. 1997; Bushman 1998; Ahmad & Mukhtar 1999; Weisburger 1999). Tea has been found to contain numerous polyphenols (flavanols /catechins/, flavonols,

flavonediols, and phenolic acids, bisflavonols, theaflavins, thearubigens, and other oligomers) and theophylline (an alkaloid of the methyl xanthine group), which may have both beneficial and hazardous effects in man. Interestingly, in most experimental studies on tea and disease, green and black tea have yielded quite similar results, and it would appear that the total polyphenol content, irrespective of the specific chemical structure, accounts for these results. At least four mechanisms of action of tea and tea polyphenols of benefit for health have been detected: 1) an antioxidative effect with beneficial effects in relation to heart disease, and some types of cancer; 2) selective induction of Phase I and II metabolic enzymes involved in the detoxification of carcinogens; 3) inhibition of cell proliferation; and 4) improved composition of the intestinal bacterial flora with beneficial metabolic actions. As phytoestrogens have been found to exert similar multiple effects, we hypothesized that tea, apart from such major constituents as flavonols and catechins, also may contain phytoestrogens that in part could account for protective properties of this beverage against cancer and atherosclerosis. Two kinds of experiments showed that green and black teas contain relatively high concentrations of lignans but only low levels of isoflavones. In experiment II (II) some of the tea samples were brewed in hot water before analysis. 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. This early liberation of the lignans from the leaves is in contrast to the great difficulties in liberating lignans from various seeds and cereals.

Numerous studies have revealed that diets low in fat and rich in complex carbohydrates from vegetables, fruits and grains are associated with a decreased risk of chronic diseases (Doll & Peto 1981; Trowell & Burkitt 1981; Steinmetz & Potter 1991a; Steinmetz & Potter 1991b;

Dragsted *et al*. 1993; World Cancer Research Fund & Research 1997; Pietinen *et al*. 1995). The frequent consumption of fresh fruits and vegetables is associated with a lower cancer and CHD incidence in human and in experimental models. This protective effect could be attributed not only to dietary fibre, but also to several active "chemopreventive" phytochemicals in plant foods, including dithiolthiones, isothiocyanates, indole-3-carbinol, allium compounds (diallyl sulfide, allyl methyl trisulfide and dipropyl trisulphide (Wargovich *et al*. 1996)), protease inhibitors, saponins, phytosterols, inositol hexaphosphate, vitamin C, D-limone, lutein, folic acid, beta carotene, lycopene, selenium, vitamin E, terpenes and flavonoids. Of particular interest as chemopreventive agents are phenolic compounds (mono/polyphenols, flavones, isoflavones, flavonoids) and tannins in foods derived from cereals, vegetables and fruits, which may be consumed in large quantities (up to 1-2 grams/day) in some human diets. Using an indirect technique of *in vitro* fermentation with human faecal microbiota, Thompson et al. (1991b) screened 68 food items for the presence of plant lignans or other precursors of mammalian ENL and END. Results showed a wide range (21 – 67,541 μg/100g sample, as-is basis) in the amount of lignans produced. Our own experiments on *in vitro* digestion and fermentation with faecal bacteria (Liukkonen *et al*. 1999) and our finding of high ENL background in the faecal inoculum as well as a compound in faeces interfering with the END assay, both of which were not recognized in the original method, suggested that the *in vitro* fermentation technique applied could consequently result in overestimation. The unknown compound interfering with END assays in methods involving blind detection systems (e.g., GC, HPLC) may occur also in other biological samples. The GC-MS method eliminates the problems of the indirect technique and gives specific evidence for the presence of the original plant lignans in foods. However, the *in vitro* technique

developed by Thompson et al. (1991) seems to have one advantage over our GC-MS method, viz. the ENL determined in that method is a product of bacterial conversion of all the precursors in foods, including also those which may not be included in our method. On the other hand, the discrepancy found between the intake of SECO and MAT with rye-based diets and faecal excretion of ENL observed in the pig study (VI) could be partly explained by the presence of other rye phytochemicals utilized by the bacteria to produce ENL in the gut (see 4.3).

The most important plant foods in the human diet are those derived from the seeds of domesticated members of the *Graminae*, the grasses - the cereals. In addition to their fibre, phytic acid and a variety of phenolic compounds (caffeic, ferrulic, gallic and ellagic acids), grains contain precursors of hormonally active mammalian lignans suggested (Adlercreutz 1984) to impart protection against such hormone-dependant diseases as breast cancer and, according to present knowledge, colon cancer (Jenab & Thompson 1996; Sung *et al*. 1998; Thompson 1998). The lignans are localized in the outer fibre-containing layers with the highest concentration in the aleurone and pericarp/testa layers (Adlercreutz 1990; Nilsson *et al*. 1997a) containing phytin, polyphenols, enzyme inhibitors and other compounds generally regarded as antinutritional factors. The 1 to 3 cell thick aleurone layer is tightly bound to the outer fibre layers, and the liberation of the lignan precursors from these very resistant cells is difficult (I). Because of its close association with the outer fibre layer, modern milling techniques usually eliminate the aleurone with the pericarp/testa layers, which are consequently seldom present in commercial products. This is particularly so in Western societies.

Cruciferous vegetables are unique in their high content of several potentially anticarcinogenic bioactive microconstituents (i.e. dithiolthiones, isothiocyanates, and indole-3-

carbinol), and their consumption has been associated with a reduction in cancer incidence. Cruciferous vegetables also contain indole-3-carbinol, which has been shown to affect estrogen metabolism in human beings (Michnovicz & Bradlow 1991; Michnovicz *et al*. 1997) and rodent models (Grubbs *et al*. 1995). Allium vegetables have been a staple of the human diet for many centuries, and they are notable for their content of the beneficial (anticarcinogenic) organosulfur components (Wargovich *et al*. 1996). The allium vegetables have been indicated to reduce risk of stomach cancer (You *et al*. 1989), colon cancer (Steinmetz *et al*. 1994) and cardiovascular disease (Warshafsky *et al*. 1994). The results of the present studies have demonstrated that the cruciferous and allium vegetables as well as other root, orange and green leafy vegetables, and berries and certain fruits, contain significant amounts of lignans (but not isoflavones) and are candidates for a preventive role with regards to chronic diseases (Adlercreutz 1990; 1999; Adlercreutz & Mazur 1997).

3. Metabolism of lignans

Knowledge of the pharmacokinetics of phytoestrogens, and of lignans in particular, is insufficient because it is mainly restricted to lignans in linseed (Morton *et al*. 1997b; Nesbitt *et al*. 1999). Factors that influence the bioavailability of lignans and concentrations of their derivatives in plasma have not been studied to any significant extent. Metabolic studies in human subjects involving lignans and measuring ENL in urine and plasma do not contain any data on the quantification of lignans in the diet consumed by subjects. Factors that might influence lignan bioavailability, such as the effect of the food matrix and the extent of intestinal bacterial fermentation and intestinal function, have been examined only in two studies on human subjects

with ileostomies (Pettersson *et al*. 1996; Hallmans *et al*. 1998) and one on subjects with jejuno-ileal bypass (Hallmans *et al*. 1998). In these studies the subjects have consumed a rye bread based high-fibre diet. In the subject with ileostomies rye bran increased the urine excretion of plant lignans, MAT, and biotransformed lignan END, but at very low concentrations. Rye bran increased the urinary excretion of MAT, END and ENL, in the subjects before jejunoileal bypass surgery. A low daily excretion of mammalian lignans was seen three months after the operation. The authors conclude from these studies that bio-transformation of plant lignans into mammalian lignans depends on a suitable bacterial microflora in the gut and that the functions of the intestinal bacteria are influenced by diet. This conclusion is in agreement with recent results of metabolic studies on isoflavonoids. It was demonstrated that the breakdown of the isoflavones by the microflora in the gut determines the recovery of the compounds, and the excretion in urine of equol and *O*-DMA is dependent on the different composition of intestinal microflora (Xu *et al*. 1995; Watanabe *et al*. 1998). Thus it could be hypothesized that the gut microflora determines modes of lignan metabolism and categorizes human subjects into lignan metabolizers, low metabolizers and non-metabolizers. Our pilot study in healthy humans gives preliminary evidence indicating that the gut microflora, influenced by diet, divides the subjects into two categories: high producers and low producers of ENL.

Statistically significant associations have been found between the intake of total fibre, fibre from berries and fruits, vegetable fibre, and legume fibre with the urinary excretion of lignans (Adlercreutz *et al*. 1987). The results of our study on lignan content in berries and metabolism of lignans in human subjects (V) are in good agreement with the correlation assessed for berries as a source of lignan precursors and urinary excretion of the chief mammalian lignan ENL. Berries,

particularly the strawberry due to its relatively high content of plant lignans, contribute significantly to plasma levels and urinary excretion of mammalian lignan ENL. The metabolic response to the strawberry meal with fixed dose of lignans was, however, different in different subjects. High individual variation of ENL values in plasma and urine was observed, before and during the experiment. There were substantial variations between the subjects in the post-challenge plasma levels and urinary excretion of the mammalian ENL. The between-individual variability of ENL levels in plasma and urine before and during the experiment is probably due to differences in individual lignan intestinal metabolism and absorption depending on the gastrointestinal microflora (Adlercreutz 1998; Rowland *et al*. 1999). It should be observed that participants (except for WM) in the study had been consuming a fibre-rich diet for many years. Production of lignans from their dietary precursors, reflected by concentration of ENL in plasma, seems to be a nonstop process stimulated by habitual high-fibre diet. There is little doubt that dietary fibre, perhaps, more than any other dietary component, has an effect on the colonic microflora and ultimately on the host (Rowland *et al*. 1985).

The urinary recovery of ENL after a single intake of strawberry meal differed only slightly from amounts estimated by its precursors in the berries, but the individual variation was large. Acknowledging the importance of the faecal excretion pathway for lignans in the human subjects, the total recovery of lignans in urine and faeces would be higher than the intake of lignans with the strawberries. This inconsistency could be explained by the enterohepatic circulation of lignans and the role of gut microflora. As microflora is an indispensable factor involved in the constant process of mammalian lignan production, the bacteria seem to be loaded with lignans and could themselves supply 5-10 % of ENL recovered in urine after the strawberry meal. The basal plasma

and urinary ENL levels, even after a long period of restricted diet, were not zero. Furthermore, the TR-FIA assay for urinary ENL gives about 30% higher values than if assayed by GC-MS (unpublished observation) probably due to the presence of other unknown lignan metabolites cross-reacting with the antiserum. The design of this study, which demanded an initial fibre-free (lignan-free) dietary regimen for at least 3 days, aimed to decrease pre-challenge bacterial fermentation and production of mammalian lignans. The diet, which was devoid of any sources of lignans such as vegetables and fruits, seeds, nuts, grains and cereals, most likely attenuated the activity of the microflora (not in every case), as indicated by low concentrations of ENL in pre-challenge plasma. This important observation - more or less making the production of lignans a dose-dependent process - is in conflict with the results of our next pig study (VI). When faecal recovery of dietary lignans was calculated in pigs fed rye bread-based high-fibre grain diets, 2-3 times as much lignans as had been ingested were recovered in the faeces of these pigs (but only half of the ingested endosperm lignans). Pig urine samples were not collected in this study and the total recovery of ENL, including ENL excreted in urine, would presumably be much higher than the value for ENL recovered separately in faeces. However, in contrast to the human strawberry study, the pigs were fed the experimental diets for 10-20 days before the collection of faecal and intestinal samples. Such a long period of intense dietary intervention with high-fibre grain diets could have triggered mechanisms resulting in quantitative and qualitative changes of intraluminal microflora, consequently leading to enhanced production of mammalian lignans. As a result, in the case of this study the samples were taken during the peak process of mammalian lignan production. The ENL recovery then was more certainly a measure of bacterial capacity and of the

potential to metabolize lignans than an absolute value measuring response to a dose of plant lignans.

A few attempts have been made to determine whether diet affects the species composition of the colonic microflora (Bornside 1978; Finegold & Sutter 1978; Hentges *et al*. 1977). When the results of these studies are taken together, no consistent, statistically significant differences emerge. However, experimental variation in these studies is quite high. Studies that have focused on particular groups of organisms have found some variation from person to person. For example, some people have much lower concentrations of bacteria which can utilize microcristalline cellulose (Betian *et al*. 1977). Bacteria which have very long generation time, such as celluloytic bacteria, may be particularly vulnerable to changes in diet which reduce residence time in the colon. On the other hand, poorly digestible dietary fibre, altering the overall composition of microflora and increasing the total number of bacteria, may induce metabolism and activity of some members of the flora that are able to utilize only this nutrient as the potential carbon and energy source. In effect, fibre-dependent species with their own specific metabolic activity become predominant in the colonic environment. Changes in nutritional status of the host (i.e. "zero diet" in the strawberry study) may alter the amount or type of substances secreted by the host into the intestine (for example, the modification of faecal bile acid concentrations by fat or dietary fibre (Heaton 1982)), thus altering bacterial substrates in the gut (Hill 1982). It is also possible that nutritional changes may alter directly or indirectly the physicochemical conditions in the gut (for example, pH of luminal contents), which in turn can affect the survival or metabolism of particular intestinal organisms.

The pig study diets, and particularly their dietary fibre, caused different fermentation patterns in the colon. While endosperm, whole rye and aleurone dietary fibre demonstrated nearly the same level of degradation (slower rate for the aleurone), the pericarp-testa dietary fibre was barely degraded. This special pattern could be due to the amount of lignin present in cell-wall material; the pericarp cells are the most lignified in the rye grain and pass through the gut more or less intact. In addition, lignin, up to 4% of rye bran and 1.1 - 3.2% of whole rye (dry matter) (Nilsson *et al*. 1997a), could play an important role by acting as a culture media component in the continuous culture analogy. The undigested lignin, together with other unabsorbed dietary fibre residues, may be regarded as providing a new microenvironment (ecological niche) for either transient organisms (acquired along with a diet from the external environment) or autochthonous flora (the true resident intestinal microbes) that are able to metabolize lignans. The other diets in the pig study contained less bran, and overall they caused a lower lignan metabolic response in the host. It may be mentioned that most vegetables and fruits contain only one-tenth the amount of lignin found in cereal grain bran (Southgate & Van Soest 1978).

A separate problem is the ileal recovery of dietary lignans ranging from 250% (the endosperm diet) to 1,050% (the pericarp-testa diet). The majority of lignans present in the ileum were conjugated plant lignans, most probably intact glucosides. In the present study, some dietary fibre components, most extensively of the endosperm and aleurone, were degraded in the ileum. This is most likely a result of the microbial activity present in the distal small intestine of the pigs (Bach Knudsen & Hansen 1991; Bach Knudsen *et al*. 1991). As shown in a study of ileostomists consuming rye bread (Pettersson *et al*. 1996), degradation of certain dietary fibre components occurred in the small intestine and was caused by the microflora present in the upper

gastrointetstinal tract (the microbial population of the ileum in ileostomists is higher than that of the normal ileum (Finegold *et al*. 1970)). The bacterial fermentation in ileostomists was not accompanied with the release of aglycones and production of mammalian lignans as judged by unchanged lignan concentrations in the plasma and urine during the high-fibre period compared with the low-fibre diet period. Nevertheless, the inconsistency between the dietary intake of the lignans and their recovery in the ileum consequently cannot be due to the activity of the microflora. A probable reason explaining this discrepancy would be an underestimation of the plant lignans in the diets using the method for food samples, either because not all precursors or intermediary forms are identified or because not all of the identified precursors are measured. As discussed above, the rigorous HCl treatment might not be radical enough to reach and liberate all lignans. Food lignans may be protected from analytical hydrolysis by the surrounding plant cell wall or by another part of food matrix. Ingested food in man (and in pigs) is primarily digested by acid hydrolysis or enzymatic cleavage by proteases, by lipases and by saccharidases found in the small intestine, which is poorly populated by bacteria. It is noteworthy that in such a way pretreated pig ileal samples (V), in which the majority of food matrix had already been digested and the microbial degradation of dietary fibre had been initiated, were analyzed by the modified food method, including acidic hydrolysis liberating both glycosides and endogenously formed conjugates (glucuronides and sulfates).

A proportion of lignans recovered in the ileal samples must have been re-excreted with the bile via the enterohepatic circulation as shown in rats by Axelson and Setchell (1981). The metabolism of estrogens, to which phenolic lignans are related, involves the enterohepatic circulation. Approximately 50-60% of circulating estrogens are conjugated in the form of

glucuronides or sulfates and are excreted in the bile (Adlercreutz 1970a; Eriksson & Gustafsson 1971; Adlercreutz & Martin 1980). Deconjugation, the prerequisite step to mucosal cell reabsorption, is catalyzed by the bacterial enzymes ß-glucuronidase and sulfatase, and is nearly complete. Indeed, 97% of the estrogens excreted in faeces is in the deconjugated form, although virtually all of the estrogens in the bile are conjugated. This seems also to be the case for lignans. The recoveries of ileal lignans showed that only 21-42% of ileal lignans were recovered in the caecum, which indicates that most of the ileal lignans were absorbed in the low ileum and in the caecum. As the ileal lignans were mostly plant lignans, two possible routes could be considered: 1) the lignans entering the enterohepatic circulation from the ileum are plant lignans absorbed as such and/or 2) absorbed mammalian lignans are re-converted by methylation in the liver into plant lignans before re-excretion into the small intestine. Plant lignans were recovered in the urine of ileostomists (Pettersson *et al*. 1996), suggesting that these are absorbed as such, whereas the re-conversion of lignans may be supported by the study by Nose et al. (1992), which indicated that the lignans arctiin and tracheloside were re-methylated in the liver of rats. Arctiin is converted to arctigenin (a structure closely related to matairesinol - having one additional methyl group) in the rat intestine (Nose *et al*. 1992) and other similar chemicals like dimeric (Han *et al*. 1994), or trimeric butyrolactone lignans could perhaps be ENL precursors. Most recently 7-OH MAT, a novel ENL precursor with chemopreventive properties, has been isolated from spruce (*Picea albieas*) (Saarinen *et al*. 1999). This may be a precursor of 7-OH ENL which was previously detected in urine (Bannwart *et al*. 1988b). Our search for new mammalian lignan precursors in various samples (e.g. *in vitro* fermentation with faecal microflora) has not yet yielded any conclusive results.

Our recent *in vitro* experiments using an artificial model of colonic fermentation suggest that rye bran lignan SECO occurs as a larger structure (Liukkonen *et al*. 1999; K. Liukkonen & W. Mazur, unpublished observation), for example in the form of oligo-/polymers as a part of a larger matrix, e.g. a lignin-phenolic acid complex (Ralph & Helm 1993; Hatfield *et al*. 1999). An earlier study by Anderegg & Rowe (1974) describing SECO polymers in the resin of parana pine knots and data on evolution of lignans formed during the course of lignification (which involves polymerization of cinnamyl alcohols) supports this view (Swain, 1986). Similar to the covalent attachment of hydroxycinnamic acids to plant cell walls (Faulds & Williamson 1999; Kroon & Williamson 1999; Hatfield *et al*. 1999), lignans could be linked to the lignin fraction or to other cell wall components via ferulic acid or diferulic bridges (cross-links) involving ester or ether linkages as shown for polysaccharides (Iiyama *et al*. 1994). The extent and type of linkages present in lignans arranged presumably in di-, tri- and/or oligomeric structures (Hatfield *et al*. 1999) would have a marked influence on lignan degradation and release of aglycones. Such a complex might be resistant to enzyme and acid hydrolysis used in the method, and lignans could not be released before reaching the colon.

An evolutionary perspective of lignification (Swain 1986) and current knowledge on lignin and lignan biochemical pathways in plants (Davin & Lewis 1996) lend support to a hypothesis of an intestinal biosynthesis of plant lignans from *p*-coumaric or ferulic acid dehydrodimers, and their further conversion to ENL. The major hydroxicinnamic acids in rye, *trans*-ferulic, *trans*-sinapic and *trans*-*p*-coumaric acids, are localized in the bran layer including the pericarp and the aleurone layer. Ferulic acid, an intermediate in the pathway to lignan formation, is the most abundant phenolic acid in rye (1 mg ferulic acid g^{-1} rye; Rybka *et al*. 1993). Ferulates appear to

act as nucleation sites for lignification (Hatfield *et al*. 1999). Reduction, demethylation of ferulic acid, and dehydroxylation at C4 are the major metabolic events carried out by microflora in the rat intestine (Scheline, 1968). Co-incubation of ferulic acid with faecal flora led to the rejection of the hypothesis of its enzymatic conversion to either of the mammalian lignans (Borriello et al 1985). In human subjects, however, the metabolic fate of dehydroferulates and other dehydrodimers cross-linking cell wall components has not been elucidated (Chesson *et al*. 1999). Breakdown of cell wall polysaccharides by microflora results in the release of dehydrodimers associated with polysaccharides, but those also incorporated into lignin are more likely to be retained in a form protected from microbial action. The ability of the gut flora to metabolize dimers once released has been little studied and may relate to the presence of several bacterial species (Chesson *et al*. 1999). The hypothesis that rye hydroxicinnamic acids may be microbially transformed to lignans remains to be tested.

SUMMARY AND CONCLUSIONS

Knowledge of the chemical composition of foods is fundamental for the planning of human nutrition. Quantitative data are essential when this knowledge is applied to prevention and management of disease and for the provision of appropriate diets for individuals and populations. A basic requirement for an understanding of the biological role of a diet-derived compound is the determination of its distribution in foods. Despite immense research efforts, the role of phytoestrogens in the maintenance of health and prevention of disease in man has not been defined. Epidemiological evidence and experimental data from animal studies are highly suggestive of the beneficial effects of phytoestrogens on human health, but the clinical data supportive of

such effects are either not available, or are awaiting design and execution of appropriate prospective large-scale clinical studies.

The four major aims of the study (p. 38) were achieved. The following summary highlights its achievements and new findings, and identifies areas where further research is absolutely necessary.

Aim no. 1.

The principal aim of this study was to develop a sensitive and specific GC-MS method for the identification and quantification of the biologically most important phytoestrogens in foods and diets. The method measures daidzein, genistein, biochanin A, formononetin, coumestrol, MAT and SECO. The method is a useful supplement to the GC-MS methods already available for phytoestrogens in human biological fluids.

Aim no. 2.

The ID-GC-MS-SIM method has been applied successfully to a wide variety of edible plants, from those with no phytoestrogens to those containing high concentrations of them. The lignan content of many foods is assessed here for the first time. Lignans, particularly SECO, are ubiquitous in plant foods but isoflavones are restricted to the Leguminosae. The detection of precursors of hormone-like mammalian lignans extends the list of biologically active phytochemicals found in legumes, vegetables, nuts, seeds, grains and teas, and may contribute to the suggested protective effects of these foods and beverages on health.

Aim no. 3.

Studies on the bioavailability of lignans could help to relate the *in vitro* effects to *in vivo* situations and lead to an understanding of the mechanisms of lignan action. The lignans can only be effective if they are metabolized, absorbed and reach tissues where they can potentially exert an effect. Studies in human subjects and pigs have yielded new information on the metabolic fate of dietary lignans. These studies have resulted in the following main observations. Berries with high lignan content contribute to plasma and urinary levels of mammalian ENL in man (V). A strawberry meal increased plasma concentration of ENL after 8-24 h and in urine in 13 - 24 h and 25-36 h urine collections. ENL excreted in the urine collected throughout the post-prandial 48h yielded on average 114 % of the plant lignans consumed. The high individual variability of the metabolic response to a single dietary lignan-rich meal observed may have important physiological and health implications for man.

Aim no. 4.

The large discrepancies between the content of plant lignans in the diets and concentrations of plant and mammalian lignans in intestinal samples and concentrations of total lignans in faeces were the important findings of the study in pigs (VI). This observation suggests underestimation of plant lignans in the rye-based diets or the presence of other ENL precursors in the food. However, in pigs a correlation was found between the level of faecal excretion of lignans and intake of dietary fibre with the rye bread-based diets (VI). As in the human study, considerable inter-individual differences in lignan metabolism were observed in pigs.

The current data on lignan metabolism in man and pigs imply that the key factor in the metabolism of lignans, perhaps also influencing their biological activities and interactions with

endogenous factors, is the colonic microflora. Undoubtedly, further studies are needed to establish the mechanisms behind the intestinal metabolism of lignans, but the present data permit a general conception of the diet- and microflora-dependent biotransformation of these plant-derived compounds. A scheme illustrating both host-related and bacteria-dependent physiological functions and degradation processes involved in the metabolism of lignans is displayed in Fig. 7. The interdependencies between diet and microflora for lignan metabolism highlighted in this study suggest the directions for the future research in the field.

The lignans represent just one of many important bio-active non-nutrients found in many plants commonly consumed in the human diet. Emerging evidence shows that lignans are compounds which, along with other phenolics and in association with dietary fibre, form a complex structure containing other phytochemicals, vitamins and minerals. Whether the lignans themselves are the active components of the dietary fibre complex in disease prevention still remains to be established. Plasma lignan levels in humans fed fibre-rich diets are lower than the concentrations found to be effective in *in vitro* studies, but these levels may already be sufficient to exert protective actions against diseases, as has already been suggested in epidemiological studies. Since fibre-rich diets contain many other phytochemicals with biological properties, a combination of these phytochemicals in low concentrations may be just as effective as a large concentration of one specific compound.

In biological fluids the lignans derived from their precursors in plants seem to be biomarkers of the intake of fibre but also biomarkers of the activity of the intestinal microflora. ENL, as the

predominant mammalian derivative of fibre-related dietary lignans, could play a role as a measure of the health-promoting potential ascribed to both the hormone-like lignans and the dietary fibre complex itself.

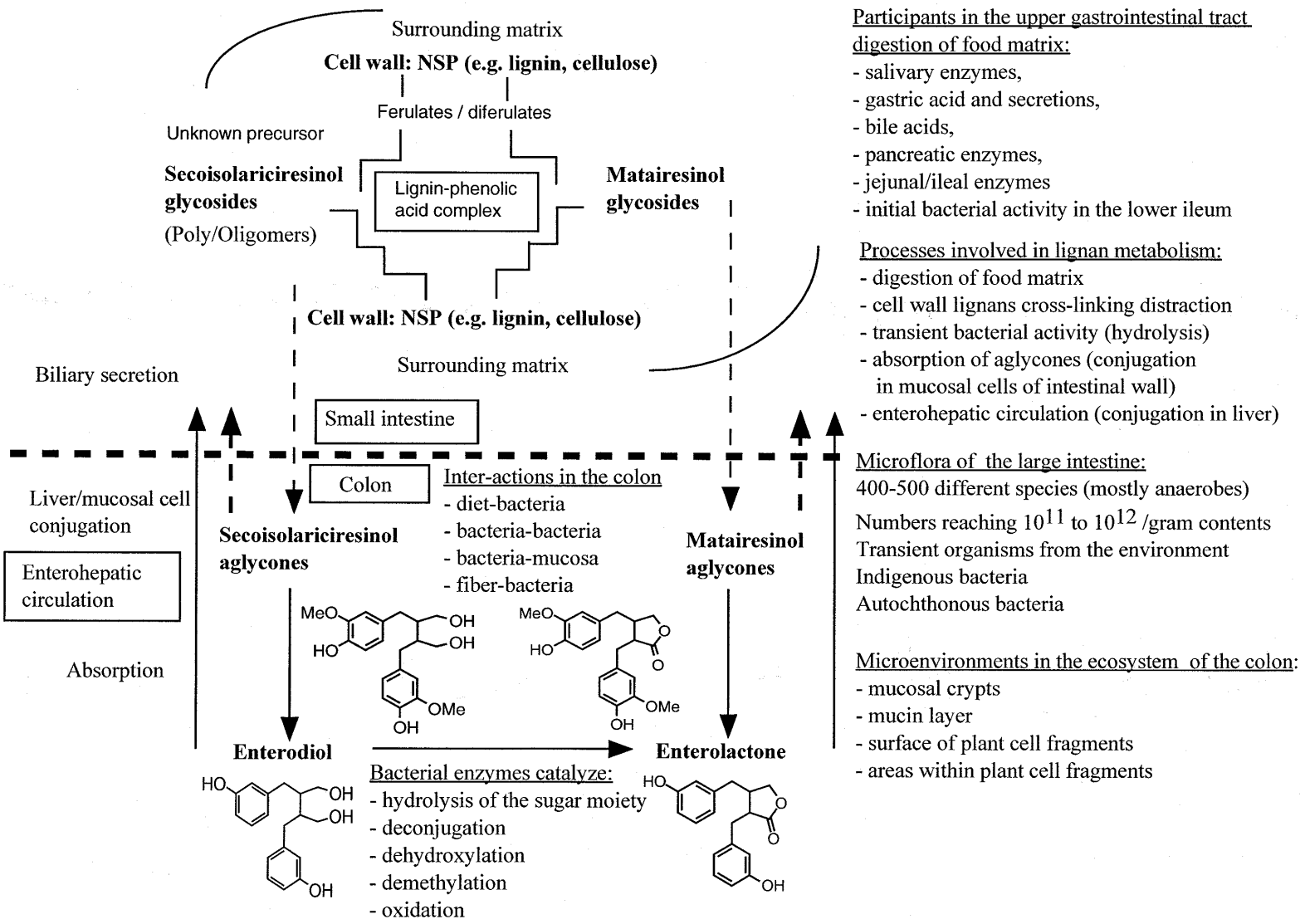


Fig. 7. Host – gut microflora interdependencies involved in biotransformation of fibre-associated dietary lignans into their hormone-like mammalian derivatives.

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