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Abstract

Plant lignans are diphenolic compounds that are present in many plant foods. The plant lignans lariciresinol (LARI), pinoresinol (PINO), secoisolariciresinol (SECO), and matairesinol (MAT) are efficiently converted into enterolignans by the intestinal microflora. Enterolignans possess several biological activities, *e.g.* antioxidant and estrogen-like activities by which they may reduce the risk of chronic diseases. Studies on the health effects of lignans were hampered by the lack of comprehensive data on the lignan contents of foods and diets.

Therefore, we developed a liquid chromatography-tandem mass spectrometry method to measure these four lignans in foods and beverages; and we constructed a lignan database with lignan contents of 83 solid foods and 26 beverages commonly consumed in the Netherlands. Almost all plant foods contained lignans. The most abundant lignan sources were flaxseed (≈300 mg/100 g; mainly SECO); and sesame seeds (≈40 mg/100 g; mainly PINO). The lignan contents of grain products, vegetables, fruits, and legumes varied mostly between 50 and 200 μg/100 g. Higher values were found for *Brassica* vegetables, garlic, French beans, apricots, strawberries, and peaches. Lignan contents in beverages ranged from 0 for cola to 91 μg/100 ml for red wine.

The median total lignan intake among a representative sample of Dutch adults was 979 μg/d (range 43-77 584 μg/d). LARI plus PINO contributed 75% to the lignan intake, whereas SECO plus MAT only 25%. Remarkably, the major food sources of lignans were beverages (37%), followed by vegetables (24%), nuts and seeds (14%), bread (9%) and fruits (7%).

Besides the consumption of lignan-rich foods, the major determinants of plasma enterolignan concentrations in an endoscopy-based population of 637 adults were defecation frequency, smoking, and body weight. The correlation between total lignan intake and plasma enterolignans was modest (r_s = 0.18).

In a prospective cohort study, in which 570 men aged 64-84 y were followed for 15 y, total lignan intake was not related with mortality. However, intake of MAT was inversely associated with coronary heart disease, cardiovascular diseases, cancer, and all-cause mortality. Multivariate adjusted rate

ratios (95% CI) per 1-SD increase in intake were 0.72 (0.53-0.98) for CHD, 0.83 (0.69-1.00) for CVD, 0.81 (0.65-1.00) for cancer, and 0.86 (0.76-0.97) for all-cause mortality. Before conclusions can be drawn, these results need to be confirmed in other prospective studies.

Keywords:
lignans, phytoestrogens, liquid chromatography, mass spectrometry, food composition, intake, cancer, cardiovascular diseases, mortality

Contents

General introduction

1

Background

A high consumption of plant foods, such as whole grain products, fruits and vegetables has been associated with a lower risk of chronic diseases.[1-4] These protective effects have traditionally been attributed to the high content of dietary fiber, vitamins and minerals in these products.[3,5,6] However, plant foods also contain numerous other bioactive compounds such as terpenes, plant sterols and polyphenols that may contribute to their protective effects.[7]

For about 20 years RIKILT-Institute of Food Safety, the National Institute of Public Health and the Environment (RIVM), and the Division of Human Nutrition of Wageningen University, in the Netherlands, have joined forces to study bioactive compounds in plant foods. This research focused on flavonoids. Flavonoids are polyphenolic compounds, which have been studied because of their antioxidant activity, by which they may prevent both cardiovascular diseases and cancer. Specific research projects have focused a.o. on flavonols and flavones[8,9] and catechins.[10]

In 2001 the 'Lignan project' was started, to study the dietary intake of lignans, their bioavailability, their molecular mechanisms of biological action, and the relation of dietary and plasma lignans with chronic diseases. The part of the project described in this thesis focuses on the dietary lignans. The studies that focused on the bioavailability and plasma lignans have been reported in the thesis of Anneleen Kuijsten.[11]

Plant lignans form a diverse group of diphenolic compounds, and have been identified in many plant foods.[12] Especially flaxseed (linseed) is a rich lignan source. After ingestion, some plant lignans can be converted to the enterolignans, enterodiol (END) and enterolactone (ENL) by the intestinal microflora, and absorbed into the body.[13] It has long been assumed that only secoisolariciresinol (SECO) and matairesinol (MAT) are precursors of enterolignans, but recently it has been shown that also lariciresinol (LARI) and pinoresinol (PINO) can be efficiently converted.[14] Enterolignans are thought to be the biologically active metabolites of plant lignans.

Besides their antioxidant activity, lignans have also gained attention because of their estrogen-like structure, which provides another mechanism by which lignan intake could modify the risk of both cardiovascular diseases and cancer. Epidemiological studies provide some evidence for the protective effects of lignan intake; however, the results are inconsistent.[15] This can be attributed to the lack of suitable methods for quantification of lignans in a wide range of foods, and consequent lack of comprehensive data on the lignan content of foods and diets.

Therefore, in the project described in this thesis, we have developed an analytical method to quantify lignans in foods, and constructed a lignan database with the four mentioned precursors. This introduction provides an overview of the occurrence of lignans in plant foods, their bioavailability, and their potential health effects in humans.

Chemical structure, biosynthesis, and potential role in plants

Plant lignans are derived from the oxidative dimerization of two phenylpropanoid (C6-C3) units.[12] The term lignan is used for optically active dimers, linked by the central carbon atoms of their side chains (FIGURE 1.1).[16] In plants, lignans can be present in their free form, but also bound to one or more sugars as glycosides, and as oligomers.[16,17] For example, in flaxseed SECO diglucoside forms oligomers with 3-hydroxy-3-methylglutaric acid via ester bonds.[18,19] The biosynthetic pathway for the formation of the plant lignans PINO, LARI, SECO and MAT in plants has been described for a few plants, including flax (*Linum usitatissimum*) (FIGURE 1.1).[20]

The role of lignans in plant physiology is still unclear. Lignans are secondary plant metabolites. They occur in roots, rhizomes, stems, leaves and fruits. Exceptionally high concentrations of lignans (6-24% w/w) have been found in wood knots of the coniferous trees (*Picea abies*).[21] Some lignans have been reported to possess antimicrobial, antifungal, antiviral, antioxidant, insecticidal and antifeeding properties.[16,22] So, they probably play a role

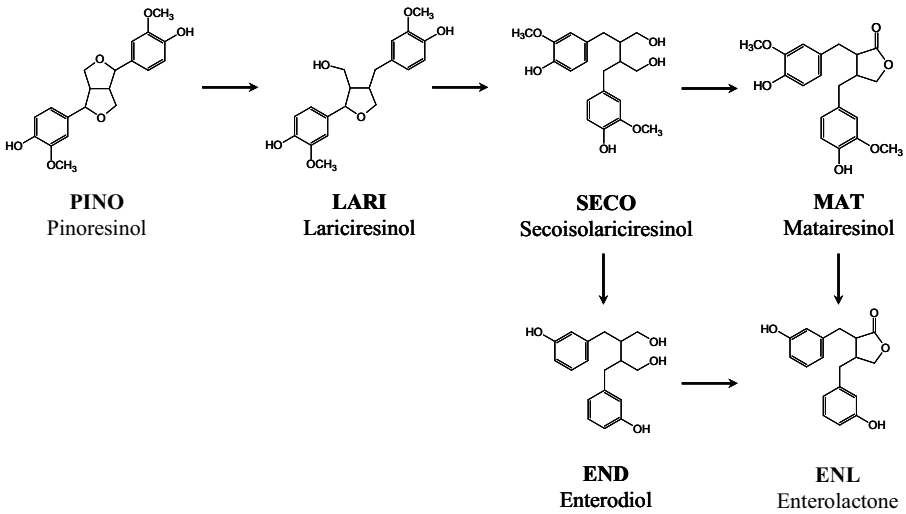

FIGURE 1.1 Proposed biosynthetic pathway of plant lignans in flax;[20] and proposed metabolic pathway for transformation of plant lignans to enterolignans by the colonic microflora.[14] The abbreviations used for plant and enterolignans throughout this thesis are also listed.

in plant defense against pathogens and pests. Furthermore, they may be involved in plant growth and development.[16]

Dietary sources and quantification

At the time of discovery of enterolignans in mammals,[23] around 1980, the presence of lignans in plants was already known, but plant lignans had not yet been established as the dietary precursors of the enterolignans. Because urinary lignan excretion is correlated with dietary fiber intake, it was hypothesized that enterolignan precursors are present in fiber-rich foods, such as grains, nuts and legumes.[24,25] The dietary origin of enterolignans could soon be confirmed, and the plant lignans SECO and MAT were identified as their precursors.[13,26] SECO and MAT were reported to be present in several seeds, and flaxseed was identified as a rich source of SECO.[13]

Thompson *et al.* were the first to report the lignan content of a range of plant foods, in 1991.[27] They did not measure plant lignans as such. Instead, they estimated the lignan content of foods from the enterolignan production after *in vitro* fermentation with a human fecal suspension. Results showed a wide range (21-67 541 μg/100 g sample) in the enterolignan production, with flaxseed being the most abundant source. Of the food groups investigated, the highest (mean ± SD) enterolignan production was found for oilseeds (20 461 ± 12 685 μg/100 g), followed by seaweeds (900 ± 247), whole legumes (562 ± 211), cereal brans (486 ± 90), legume hulls (371 ± 52), whole grain cereals (144 ± 23), vegetables (144 ± 23) and fruits (84 ± 22).

All other researchers have quantified SECO and MAT directly in foods. Mazur *et al.* were the first to developed an analytical method for the quantification of these lignans in foods.[28] In this method, lignans are released using acid and enzymatic hydrolysis, and quantified using GC-MS. Subsequently, they have reported lignan contents of a variety of Finnish foods on a dry weight basis (μg/100 g dry weight).[28-33] In general the amount of SECO in foods was higher than that of MAT. The amount of SECO was very high in flaxseed, and also the amount of MAT was relatively high.[31] Concentrations in other oilseeds such as, sesame, clover, sunflower, caraway, poppy, and peanut were much lower. Lignan concentrations in whole grain were 48-112 μg/100 g. In grain brans the concentrations were higher than in whole grain 63-299 μg/100 g; whereas in flour they were lower 8-32 μg/100 g. The total lignan concentrations for nuts were 96-261 μg/100 g, for vegetables 16-3874 μg/100 g, for fruits and berries 5-1510 μg/100 g, and for legumes 0-476 μg/100 g. In addition, relatively high lignan concentrations were reported for tea leaves 770-3050 μg/100 g, and coffee powder 393-716 μg/100 g.[33]

Horn-Ross *et al.* have quantified SECO and MAT in 112 American foods, by HPLC-MS.[34] They have reported lignan contents on a wet weight basis. Because of the relatively high detection limit (25 μg/100 g) of their method, they could detect lignans in only 14 of the 112 foods. They found relatively high (> 100 μg/100 g) lignan values in dried apricot, soybean, and sunflower seeds. Lignan values between 25 and 100 μg/100 g were reported for a few soy products (soy milk, canned chili, English muffins with added soy flour, and soy burgers), vegetables (asparagus, carrots, cauliflower, garlic, and sweet potatoes), and fruits (peaches, prunes, and raisins).

At the start of the Lignan project, the presence of LARI and PINO had been reported for a few specific foods, such as olive oil[35], sesame seeds,[36] and flaxseed,[37,38] but other than that, no data on the presence of these lignans in foods were available.

Bioavailability and metabolism

Conversion of plant lignans to enterolignans

After ingestion, a small fraction of the plant lignans may be absorbed as such in the small intestine.[39] However, the majority of the lignans can not be absorbed there, because they first have to be released from the food matrix and the glycosides and oligomers in which they occur. Although some lignan glycosides may be deconjugated by gastric acid and intestinal β-glycosidases, the majority reaches the colon intact.[39] In the colon, they can be deconjugated by bacterial β-glycosidases, and converted to enterolignans by a series of metabolic reactions. SECO can be converted to END, and MAT to ENL (FIGURE 1.1).[13,40] END can be further oxidized to ENL. The metabolic pathway for conversion of PINO and LARI to enterolignans is supposed to be similar to the biosynthetic pathway in plants (FIGURE 1.1).[14,41] Thus, PINO may be converted in enterolignans via LARI and SECO. In addition to those four plant lignans, also other plant lignans, such as syringaresinol,[14] arctigenin,[14] 7-hydroxy-MAT,[21] and sesamin[42,43] have been identified as enterolignan precursors, but they had low degrees of conversion (<15% vs. 55-100%).[14]

The important role of the intestinal microflora in the conversion of plant lignans to enterolignans has been established by the negligible enterolignan production in germ-free rats.[44] It has been confirmed by studies in humans that showed that urinary and serum enterolignan concentrations are severely reduced after antibiotic use, which can partly destroy the intestinal microflora.[45,46] In recent studies, a few bacterial strains capable of one or more of the metabolic steps required for the conversion of plant lignans to enterolignans have been identified.[41,47-49]

Absorption, metabolism and excretion

Lignans may be directly excreted from the colon via feces, or they may be absorbed into the blood stream. Enterolignans are conjugated into glucuronides, sulfoglucoronides, monosulfates and disulfates in the intestinal epithelium[50] or in the liver.[51] The usual plasma concentration of enterolignans in Western populations is reported to be in the range of 10-30 nM.[11] However, values up to 1000 nM have been reported for specific groups, such as vegetarians, and after intervention with lignan-rich products. Plant lignans have also been identified in plasma, but at low concentrations compared with the enterolignans. [52]

Via the blood, lignans may be transported to several tissues. Enterolignans have been identified in diverse body fluids, such as prostatic fluid,[53] semen,[54] and amniotic fluid.[55] Data on plant lignans in these fluids are not available. A part of the absorbed enterolignans may be re-excreted in the intestinal tract via bile, and subsequently leave the body via feces, or be reabsorbed into the bloodstream, and thus undergo enterohepatic circulation. Ultimately, lignans are excreted via the urine. [56-58]

Biological activities

Antioxidant activity

The antioxidant activity of some plant lignans and of the enterolignans has been evaluated in several *in vitro* test systems. SECO diglucoside, ENL and END were demonstrated to possess antioxidant activity in various test models at concentrations ranging from 10-100 μM.[59] They did not show to have prooxidant activity in this concentration range. In the FRAP (Ferric Reducing Antioxidant Power) assay, SECO and MAT had high antioxidant activity compared to ascorbic acid.[60] However, the enterolignans were far less active in this assay. Willfor *et al.* showed that SECO, MAT, hydroxy-MAT, and LARI had high potency to inhibit lipid peroxidation *in vitro*.[61] In addition, they could scavenge superoxide and peroxyl radicals. The IC_{50} (concentration showing 50% inhibition) values in these assays were between 2.9 and 126 μg/L. In an assay of the chemiluminescence of zymosan-activated polymorphonuclear leukocytes SECO diglucoside, SECO, END, ENL, and vitamin E, in the concentrations between 0.5 and 10.0 mg/mL, showed antioxidant activity.[62] The order of antioxidant activity was SECO = END > ENL > SECO diglucoside = vitamin E. PINO and acetoxy-PINO, the major lignans in olive oil, were reported to be potent antioxidants based on their inverse relation with xanthine oxidase activity and reactive oxygen species concentration in an fecal assay.[63] Pool-Zobel *et al.* showed that ENL was able to reduce the endogenous gener-

ation of oxidized DNA bases.[64] However, END and ENL were not able to reduce hydrogen peroxide induced DNA damage in HT29 colon tumor cells at a concentration of 100 μM nor did ENL reduce intracellular oxidative stress.

In summary, both plant and enterolignans are relatively potent antioxidants *in vitro*. In some assays, the plant lignans had clearly higher antioxidant activity than the enterolignans, whereas in other assays, they had similar antioxidant activities. It is difficult to translate the results of *in vitro* studies to the *in vivo* situation, especially since in many assays the concentrations used, and the form of the lignans (*e.g.* unconjugated enterolignans, SECO diglucoside) did not reflect the *in vivo* situation.

So far, two intervention studies investigated the antioxidant effects of lignan intake in humans, and the results did not support the *in vitro* results.[64,65] In a study with rye bread, no effect on genetic damage in blood lymphocytes or plasma antioxidant activity was found, but probably the lignan intake in this study was too low (< 100 μg of SECO + MAT /d).[64] Also in a study using a lignan complex isolated from flaxseed, delivering approximately 500 mg/d of SECO diglucoside, no effects on serum lipoprotein oxidation resistance and plasma antioxidant activity were found.[65]

Estrogen-like activity

The structure of lignans is similar to that of endogenous estrogens, such as 17-β estradiol, and thus lignans are classified as phytoestrogens. Two other important classes of phytoestrogens are the isoflavones and the coumestans.[66] Both enterolignans[67] and plant lignans[68] may bind to the estrogen receptors α and β, but at low affinity compared to endogenous estrogens. They may act as a weak estrogen agonist or antagonist depending on the endogenous estrogen concentration. However, most studies, only evaluated the (anti)estrogenic effects of the enterolignans, and not of the plant lignans.[69]

Enterolignans were shown to decrease the proliferation of several human cancer cell lines *in vitro*.[69-71] In addition, enterolignans may affect enzymes involved in the formation of estrogens,[72] such as aromatase,[68,73] and 5α-reductase,[74] and may stimulate the production of sex hormone-binding globulin (SHBG),[68] which may subsequently decrease free estradiol.

In rats, flaxseed and SECO diglucoside could induce lengthening or cessation of the estrous cycle, which also provides further evidence of the estrogenic effects.[75] In humans, intervention with flax has been shown to modify the ratio of urinary estrogen metabolites in postmenopausal women.[76]

Other

In addition to their antioxidant and estrogen-like activity, a few other potential mechanisms for the protective effects lignan intake have been identified. For instance, ENL was able to stimulate quinine reductase, a phase II detoxification enzyme,[77] and MAT could inhibited matrix metalloproteinase 7 (MMP-7), which is thought to play a role in tumor invasion and metastasis.[78] SECO diglucoside, was shown to reduce total serum cholesterol and atherosclerosis in rabbits,[79] and it had antihypertensive[80] and angiogenic activity in rats.[81] In several human intervention studies, consumption of flaxseed could reduce total and LDL cholesterol, without an influence on HDL or total triglycerides.[82] However, besides lignans flaxseed also contains relatively high amounts of α-linolenic acid and soluble fiber, so it is not clear whether these results can be attributed to the lignans present in flaxseed.

Relation of dietary lignans with cardiovascular diseases and cancer

Animal studies

The effect of lignan intake on cardiovascular diseases has hardly been evaluated in experimental animals. We could identify only one study, in which the intake of SECO diglucoside was evaluated in a myocardial infarction rat model. In this study, supplementation with 20 mg SECO diglucoside/kg body weight for two weeks, decreased the infarct size, the extent of apoptosis, and could improve angiogenesis.

The effects of lignan-rich foods such as flaxseed, or isolated lignans, on mammary, prostate, and colon cancer has been evaluated in several animal experiments.[70,83] In general, these studies showed protective or neutral effects for breast and colon cancer, whereas for prostate cancer the results were not convincing.[70] Here we report only on the results for isolated SECO and MAT. No studies reported on isolated LARI and PINO.

Most studies have reported on SECO diglucoside, which can be deconjugated to SECO in the intestinal tract. SECO diglucoside at 1.5 mg/d reduced the dimethylbenz(a)anthracene (DMBA) induced formation of mammary cancer in rats.[84,85] However, in N-methyl-N-nitrosuria treated rats, SECO diglucoside gave conflicting results; at low doses it promoted, whereas at high doses it inhibited tumor multiplicity.[86] SECO diglucoside prevented metastasis of melanoma cells in mice, although only at a high concentration equivalent to 10% flaxseed in the diet. It decreased the number of aberrant crypts in the colon of carcinogen-treated rats.[87] However, isolated SECO at a level of 0.02% in the diet (w/w) did not prevent intestinal tumor formation.[88]

Only one study reported on the effects of isolated MAT. In this study MAT at 0.02% in the diet did not prevent intestinal tumor formation in Apc Min mice, an animal model for adenomatous polyposis colimultiple intestinal neoplasia.[88] In fact, the mice fed MAT had larger tumors, and thus MAT may even promote tumor growth. Although 7-hydroxy-MAT is not metabolized to MAT, we also report the results for 7-hydroxy-MAT, because of its structural resemblance to MAT and because it can be metabolized to ENL.[89] 7-hydroxy-MAT was shown to prevent intestinal tumor formation in Apc Min mice.[90] In addition, it could inhibit DMBA-induced mammary tumors,[21,91,92] the growth of human prostate cancer xenografts in athymic mice,[93] and the development of uterine adenocarcinoma in carcinogen-treated rats.[94]

Epidemiological studies

The epidemiologic evidence on the potential protective effects of lignan intake as well as high plasma or urinary lignan concentrations, has recently been reviewed.[11] Here we highlight the dietary lignan intake studies. Previous studies on dietary lignan intake have only included SECO and MAT. Alternatively, in some studies, lignan intake was estimated from the enterolignan production after *in vitro* fermentation of foods.

So far, only one study has reported on the relation between lignan intake and cardiovascular disease risk.[95] In this prospective study, lignan intake was not related with coronary or cerebrovascular events in women, although a small risk reduction of coronary events was observed in smokers. In addition, in a few cross-sectional studies the relation between dietary lignan intake and risk factors for cardiovascular diseases was examined.[96-100] Of the risk factors studied, only plasma triglycerides, waist-hip ratio, and the metabolic syndrome score, a summary score in which several risk factors are combined, were inversely associated with dietary lignan intake in women.[101] In addition, aortic stiffness was inversely associated with lignan intake in women, but this was only statistically significant for women with a postmenopausal time span of 20 years or more. In men, dietary lignan intake was significantly inversely associated with plasma insulin, and C-peptide, whereas LDL-cholesterol and apoB were positively associated.[100]

The relation between lignan intake and cancer has mainly been studied with respect to hormone-related cancers, particularly breast cancer. Five case-control studies have reported an inverse association between lignan intake and breast cancer risk,[102-106] although in one study the association was not statistically significant,[106] and in one study it was significant only in premenopausal women with estrogen receptor negative tumors.[103,107] The

observed risk in the highest category of lignan intake in these studies was about 30-50% lower. In two of these case-control studies, a modifying effect of the CYP17 genotype was reported.[102,104,108] In these studies, the observed risk reduction was only significant in women with at least one,[102] or two CYP17A2 alleles.[108] In one case-control study there was no association between lignan intake and breast cancer.[109]

Although lignan intake was associated with a lower breast cancer risk in case-control studies, this was not the case in prospective studies.[109,110] In one of these studies breast cancer was significantly positively associated with intake of SECO, but not with intake of MAT.[110] In an other study, no association between lignan intake and breast cancer was observed.[109]

Besides with breast cancer, lignan intake was also inversely associated with risk of ovarian cancer,[111] thyroid cancer,[112] and endometrial cancer[113] in case-control studies in women. Case-control studies in men showed that lignan intake was not associated with testicular cancer[114] or prostate cancer.[115-117] In both men and women, lignan intake was inversely associated with colorectal cancer[118] and lung cancer.[119] However, for women the lower risk for lung cancer was only significant when lignan intake was estimated from the enterolignan production of foods, and not when this was estimated from the intake of SECO plus MAT, whereas in men both methods yielded significant lower risks.[119]

Research aims and outline of this thesis

In summary, experimental and animal studies have provided several mechanisms by which lignans could protect against both cardiovascular diseases and cancer. Epidemiological studies on lignan intake have been hampered by the fact that data on the lignan content of foods and diets were limited. Thus, the main aims of the research described in this thesis were to provide comprehensive data on the lignan content of foods and diets in the Netherlands, and to evaluate the relation between dietary lignan intake and cardiovascular diseases and cancer.

At the start of the project, methods for the quantification of all four major plant lignans, in a wide range of foods, were not available. Therefore, we first developed and validated an analytical method for the quantification of these lignans in foods and beverages (CHAPTER 2). Subsequently, we constructed a lignan database including lignan contents of commonly consumed plant foods in the Netherlands (CHAPTER 3). Using this database we determined the habitual intake and major dietary sources of lignans in the Netherlands (CHAPTER 4). In addition, we evaluated the relation between

intake of dietary lignans and plasma lignan concentrations in an endoscopy-based population (CHAPTER 5). Finally, we evaluated the relation between lignan intake and cardiovascular and cancer mortality in a prospective cohort study of Dutch elderly men (CHAPTER 6). We conclude with a discussion of the main findings of this thesis (CHAPTER 7).

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An analytical method for the quantification of four plant lignans in foods and beverages

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2

Abstract:
A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed for the quantification of the four major enterolignan precursors secoisolariciresinol (SECO), matairesinol (MAT), lariciresinol (LARI) and pinoresinol (PINO) in foods. The method consists of alkaline methanolic extraction, followed by enzymatic hydrolysis using *Helix pomatia* β-glucuronidase/sulfatase. *H. pomatia* was selected from several enzymes based on its ability to hydrolyze isolated lignan glucosides. After ether extraction samples were analyzed and quantified against d_8-SECO and d_6-MAT. The method was optimized using model products: broccoli, bread, flaxseed, and tea. The yield of methanolic extraction increased up to 81%, when it was combined with alkaline hydrolysis. Detection limits were 4-10 μg/100 g dry weight for solid foods, and 0.2-0.4 μg/100 mL for beverages. Within- and between-run coefficients of variation were 6-21% and 6-33%, respectively. Recovery of lignans added to model products was satisfactory (73-123%), except for MAT added to bread (51-55%).

Keywords:
lignans, HPLC-MS/MS, phytoestrogens, secoisolariciresinol, matairesinol, lariciresinol, pinoresinol

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Introduction

Lignans are polyphenolic compounds in plants derived from the combination of two phenylpropanoid (C6-C3) units.[1] Several hundred lignans have been discovered in plant species. A small number of these plant lignans are known to be converted by the colonic microflora into the 'enterolignans' enterodiol and enterolactone. Enterolignans are present in biological fluids of humans and animals.[2-4] Enterolignans possess several biological activities such as antioxidant, antitumor and (anti-)estrogenic, and inhibit enzymes involved in the metabolism of sex hormones (*e.g.* SHBG, 5α-reductase, 17β-hydroxysteroid dehydrogenase.[5-10] Because of these activities, they may affect the development of hormone-dependent diseases.[11,12] Increased plasma or serum levels or urinary excretion of enterolactone were associated with reduced breast cancer risk in epidemiological studies.[13-15] Only one of these studies also included enterodiol,[14] with similar results. However, increased risks were also found.[15,16] There also is some support for a protective effect of lignans against cardiovascular diseases. Negative associations have been found between serum enterolactone and acute coronary events and cardiovascular disease related mortality.[17,18] Besides a negative association between serum enterolactone and F_2-isoprostanes, a marker of *in vivo* lipid peroxidation, was found in a cross-sectional study in men.[19]

Until recently plant lignan research focused on secoisolariciresinol (SECO) and matairesinol (MAT) (FIGURE 2.1), because they were thought to be the only enterolignan precursors. SECO and MAT are present in grains, seeds, vegetables and fruits.[20,21] Flaxseed contains by far the highest concentrations of SECO of any food for which data have been published.[20,21] In some human and animal supplementation trials with rye bread or wheat bran the enterolignan excretion was much higher than expected based on the amount of SECO and MAT consumed.[22-24] Heinonen and co-workers showed that also lariciresinol (LARI) and pinoresinol (PINO) (FIGURE 2.1) were converted into enterodiol and enterolactone after *in vitro* fecal incubation (55 and 100% conversion, respectively).[23] In addition, syringaresinol, arctigenin and 7-hydroxy-MAT were metabolized to enterolignans, but to a smaller extent (4-15% conversion). These newly discovered lignan precursors might explain the difference between lignan intake and excretion in the aforementioned trials, and indeed some of these precursors have been detected in rye bran.[23] It is important to include these newly discovered enterolignan precursors, especially LARI and PINO, in studies that assess the health effects of dietary lignans.

For the analysis of SECO and MAT in foods several methods have been published,[25] but quantitative methods for LARI and PINO are lacking. To enable

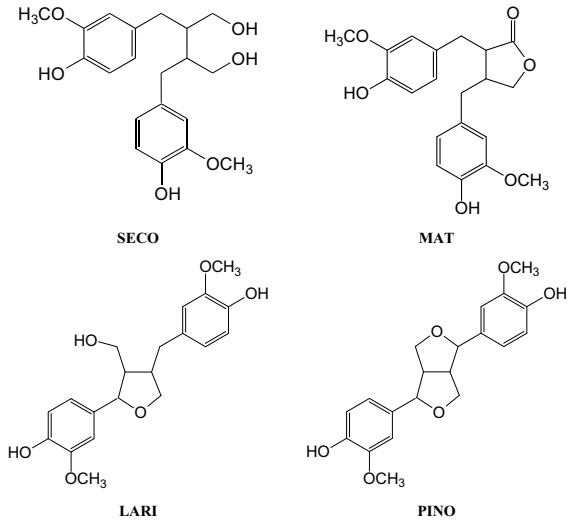

FIGURE 2.1 Chemical structures of plant secoisolariciresinol (SECO), matairesinol (MAT), lariciresinol (LARI), and pinoresinol (PINO).

quantification, lignans have to be extracted from the food matrix. Extraction is usually performed using organic solvents, acid or alkaline hydrolysis, or combinations of these. Alkaline hydrolysis is mainly used to liberate SECO diglucoside from flaxseed,[26-28] since SECO in flaxseed forms oligomers with 3-hydroxy-3-methylglutaric acid via ester bonds, which can be hydrolyzed using alkali. However, we discovered that alkaline hydrolysis also improved lignan yield from other products (this paper). Plant lignans occur bound to one or more sugars as glycosides. Because not all glycosides are known, lignans are usually measured after hydrolysis to aglycones. This can also be achieved with acid hydrolysis, or with enzymatic hydrolysis. Liggins *et al.* showed that optimal acid hydrolysis times vary with the food matrix.[29] In addition, aglycones appeared to be unstable during hydrolysis. Therefore we decided to pursue an enzymatic method. Enzymatic methods include hydrolysis with β-glucosidase[30] or *Helix pomatia* β-glucuronidase/sulfatase.[31,32]

Published analytical techniques for the measurement of lignans in foods include reversed phase high performance liquid chromatography (RP-HPLC) with ultraviolet (UV)[26,31] or coulometric electrode array detection,[28,33,34] gas chromatography with mass spectrometric detection (GC-MS),[29,35] or liquid chromatography with mass spectrometric detection (LC-MS).[30,31] HPLC-UV is used for quantification of SECO in flaxseed. Because of its limited sensitivity and specificity it is not suitable for products with low lignan values. Coulometric detec-

tion offers adequate sensitivity, but proved to be not selective enough for a wide range of foods. A disadvantage of GC-MS is that it requires time-consuming cleanup and derivatization, with consequent risk of losses. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) requires no derivatization and is both specific and sensitive.

Here we present a quantitative method for the determination of the plant lignans LARI, PINO, SECO and MAT in foods. This method consists of combined extraction and alkaline hydrolysis (for solid foods), followed by enzymatic hydrolysis using *Helix pomatia* β-glucuronidase/sulfatase. After a subsequent ether extraction, lignans are separated and detected using LC-MS/MS and quantified against deuterated standards of SECO and MAT.

Materials and Methods

Chemicals

Pure standards of SECO (purity *ca.* 92%) and MAT (purity > 98%) were obtained from Plantech (Reading, England). Enterolactone was purchased from Fluka Chemie GmbH (Buchs, Switzerland). LARI isolated from the wood of *Abies sachalinensis* and PINO and PINO diglucoside isolated from *Euconmia ulmoides oliv*. bark were kindly provided by Dr. Ozawa (Rakuno Gakuen University, Japan), Dr. Deyama (Yomeishu Seizo Co., Ltd., Japan) and Dr. Nishibe (University of Hokkaido, Japan). SECO diglucoside isolated from flaxseed (purity 93%) was purchased from Dr. P. Winterhalter (Technical University of Braunschweig, Germany). d_8-SECO and d_6-MAT with isotopic purity >98%[32] were synthesized by Dr. K. Wähälä. β-Glucuronidase/sulfatase (*Helix pomatia*), β-glucuronidase (bovine liver) and β-glucosidase (almonds) were purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands). Rapidase LIQ$^+$ was obtained from DSM Food Specialties (Seclin, France). All other chemicals were of analytical grade, and water was purified with a Milli-Q system.

Sample pretreatment

Preparation of test products. All test products used to optimize the method were bought at a local supermarket and prepared the same day. Wholegrain wheat bread was cut into pieces, frozen under liquid nitrogen and freeze-dried the same day. Flaxseed was ground to a powder using a Retsch GP 200 mill (Labotech BV, Ochten, the Netherlands) for 15 s at 8000 rpm and stored at –20°C until analysis. Broccoli was chopped into pieces under liquid nitrogen, and freeze-dried the same day. After freeze-drying, broccoli and bread were ground to a powder and stored at –20°C until analysis. Black tea from tea

bags was passed through two sieves of 0.8 and 0.355 mm, and the middle fraction was stored at room temperature for use as a test product. For each series of analysis fresh tea was prepared by adding 100 mL of boiling tap water to 1 g of tea. After 5 min it was stirred, and 50 mL was filtered through a 1.2 µm Acrodisc filter (Gelman Sciences, Ann Arbor, USA).

Analytical scheme. Alkaline extraction of 1.0 g of dry food was performed with 24.0 mL methanol/water (70% v/v) containing 0.3 M sodium hydroxide, in a shaking water bath for 1 h at 150 rpm and 60°C. After extraction, the pH was adjusted to 5-6 with 750 µL 100% glacial acetic acid (Supra Pure) and the extract was centrifuged for 10 min at 10°C and 4500 g. An aliquot of 1 mL was transferred to a pre-weighed test-tube. Methanol was evaporated from this aliquot using a Zymark Turbo vap LV Evaporator (Zymark Corp., Hopkinton, MA) at 60°C, under a mild nitrogen flow (5-10 psi), until the residual weight of the extract was ≤ 0.30 g. Then the volume was adjusted to approximately 1.2 mL with sodium acetate buffer (0.05 M, pH 5.0), and the extract was weighed again, in order to calculate the dilution compared to the original aliquot of 1 mL.

A 1 mL aliquot of this weighed extract or 1 mL beverage was hydrolyzed by the addition of *Helix pomatia* β-glucuronidase/sulfatase (0.83 mg, in 1 mL 0.05 M sodium acetate buffer, pH 5.0). The samples were incubated overnight at 37°C. Samples were extracted twice with 2 mL diethylether and the two organic phases were combined. The diethylether was evaporated using a Turbo vap at 30°C, under a mild nitrogen flow (5-10 psi). The dried samples were redissolved in 0.3 mL methanol, mixed and 0.7 mL water was added. 240 µL of sample was added to 10 µL internal standard solution containing 50 ng d_8-SECO and 50 ng d_6-MAT in 30% aqueous methanol. Samples were mixed and transferred to HPLC vials. Samples were analyzed the same day or stored at –80°C (for a maximum of one week) until analysis.

Chromatographic and detection conditions

Chromatographic conditions. A Waters (Millford, MA) Alliance chromatography separation Module 2690 was used, which consisted of a chromatographic system, and an autosampler with a cooled sample tray which was set at 10°C. Separations were performed on a 150 mm × 3.0 mm i.d., 5 µm Symmetry C18 column (Waters), at a flow rate of 0.4 mL/min at 40°C. The mobile phases A and B consisted of water and methanol respectively. The gradient was as follows: 0-0.5 min, 30% B; 0.5-12 min, linear gradient from 30 to 50% B; 12-15 min, isocratic at 50% B; 15-15.2 min, linear return to 30% B, 15.2-

19 min, isocratic at 30% to equilibrate. The total run time for each sample was 19 min. The sample injection volume was 50 µL. The divert valve was programmed to allow flow into the mass spectrometer from 8 to 19 min of each run.

Detection. Detection was performed with a Micromass Quatro Ultima MS, (Micromass, Manchester, UK) equipped with an Atmospheric Pressure Chemical Ionization (APCI) source, operated in the negative ion mode. Th cone voltage was set at –30 V. Nitrogen at a flow rate of 100 L/h was used as desolvation gas. Source and desolvation temperatures were set at 100 and 500°C, respectively. Dwell time was set at 0.2 s for all the compounds. For quantification of lignans, the deprotonated molecules were used as precursor ions and the most abundant fragments after collision-induced association were selected as product ions. Argon was used as collision gas at a pressure of 2.2×10^{-3} mbar The retention times, characteristic precursor/product combinations and collision energies for each compound were as follows: LARI, 9.6 min, (359.1/329.1), 12 eV; SECO, 9.9 min, (361.1/165.1), 23 eV; PINO, 12.2 min, (357.1/151.2), 12 eV; MAT, 12.9 min, (357.1/83.1), 20 eV; d_8-secoisolarciresinol, 9.7 min, (369.2/168.3), 25 eV and d_6-MAT, 12.8 min, (363.2/83.1), 25 eV. A second, less abundant ion was selected for confirmation. However, in samples confirmation was not always possible because of lack of sensitivity. Integration of peak areas was performed using the program Masslynx provided by the MS manufacturer.

Method validation

Calibration and quantification. Calibration standards with lignan concentrations of 20, 100, 200, 600 and 1000 ng/mL plus 200 ng/mL of the deuterated standards in 30% aqueous methanol were injected three times (at the beginning, middle and end of each series of analyses). Calibration lines were obtained by plotting the response factor (area lignan/area deuterated standard) against the concentration of the calibration standard. Average calibration lines were constructed from all of the injections. d_8-SECO was used to calculate the response factor of SECO and LARI and d_6-MAT was used for MAT and PINO, since these lignans elute close to those deuterated standards (FIGURE 2.2). All calibration lines were forced through the origin, with correlation coefficients ≥ 0.99.

When MS is used for quantification, signal enhancement or suppression can occur, causing over- or underestimation of the actual concentrations. This is caused by interferences introduced by the food matrix. To adjust for these matrix

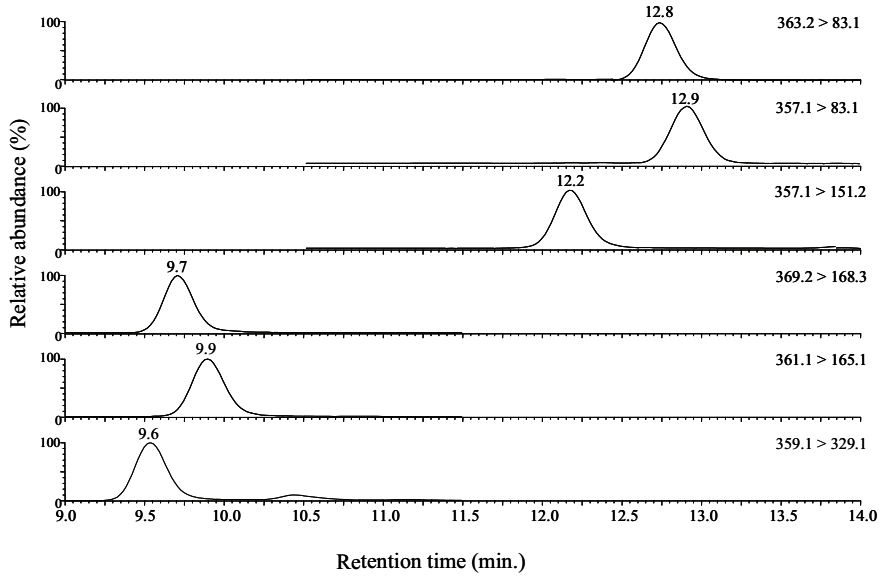

FIGURE 2.2 Individual chromatograms of a typical analysis of tea. The chromatograms show the MRM transitions that were used for quantification. From bottom to top, LARI (359.1>329.1) and SECO (361.1>165.1) with internal standard d_8-SECO (369.2>168.3), PINO (357.1>151.2) and MAT (357.1>83.1) with internal standard d_6-MAT (363.2>83.1).

effects, 200 ng/mL of deuterated standards was added to each sample, and lignan concentrations were calculated on the basis of the response factor (area lignan/area deuterated standard). To establish whether this adjustment was sufficient, calibration lines with 0, 20, 100, 200, 1000 and 2000 ng/mL of all lignan standards were made in 30% aqueous methanol or in food extracts (tea, broccoli, and bread). Each of the calibration standards was injected two times, and the average response factor was used to construct the calibration lines. The slopes of calibration lines with and without food matrix were compared. If the adjustment is sufficient, the slopes of the calibration lines in food extracts and calibration lines in aqueous methanol are equal, even though the intercepts increase when lignans are present in the food extracts. In addition, the effect of dilution of the food extracts was evaluated using bread. Calibration lines with 0 to 2000 ng/mL of isolated lignans added were constructed in 3.3 x concentrated, undiluted and 2 x diluted extract. Each calibration standard was injected three times.

Limits of detection. Limits of detection were determined by triplicate injections of 50 µL of standard mixtures, containing 20 ng/mL of each lignan standard, on five different days. The limit of detection was defined as the

amount of lignan resulting in an extrapolated signal to noise (S/N) ratio of 3, average S/N ratios from five days were used.

Recovery. Recovery of lignans was determined by spiking known amounts of lignan standards to bread (total method) and tea (method without extraction). For bread the standards were added immediately after the addition of extraction solvent, and for tea immediately after filtering the brew and cooling down to room temperature. Lignan standards were added at approximately 100 and 200% of the original level in bread, and 50 and 100% of the original level in tea. Because MAT was not present in well detectable amounts, it was added at two higher levels.

Precision. To determine within- and between-run variation of the method, duplicate analyses of a tea, broccoli, and bread control sample were carried out on four to seven separate days.

Stability. The stability of sample extracts was evaluated, to enable storage for up to one week at –80°C before analysis. Seven sample extracts (together containing all four lignans) were analyzed before and after storage at –80°C for 9 days. The concentrations were compared using ANOVA for repeated measurements for statistical analysis.

Results

Sample pretreatment

Alkaline extraction. The combined extraction and alkaline hydrolysis was optimized using broccoli and bread as test products. Broccoli and bread were extracted for 3 h using 0, 0.02, 0.05 and 0.20 M sodium hydroxide, at 20 and 60°C. For both products the yield of lignans was higher at 60 than at 20°C, irrespective of the sodium hydroxide concentration (results not shown). For broccoli the influence of the sodium hydroxide concentration on the yield of lignans was less than 25% (results not shown), but bread required further optimization. The lignan yield was optimal at 0.3 M sodium hydroxide, when an extended range of 0, 0.05, 0.1, 0.2, 0.4 and 1 M sodium hydroxide was tested (FIGURE 2.3). Additional influence of the hydrolysis time (0.5, 1, 2, 3, 4 h) at 0.3 M sodium hydroxide was less than 20% (results not shown). Thus 1 h extraction with 0.3 M sodium hydroxide at 60°C was chosen for the final method.

Subsequently, the effects of omitting the alkaline hydrolysis from the method were evaluated. For flaxseed and bread, omitting the alkaline extraction

TABLE 2.1 Lignan yield without alkaline hydrolysis compared to method including alkaline hydrolysis.

	Percentage yield*			
	LARI	PINO	SECO	MAT
Flaxseed	51 ± 4	46 ± 5	19 ± 2	35 ± 1
Broccoli	78 ± 10	98 ± 5	84 ± 12	-
Bread	55 ± 7	49 ± 3	39 ± 2	-
Tea	95 ± 1	110 ± 4	99 ± 16	420 ± 12

*Results are expressed as a percentage of the yield of the method including alkaline hydrolysis (mean of duplicate analyses ± SD).

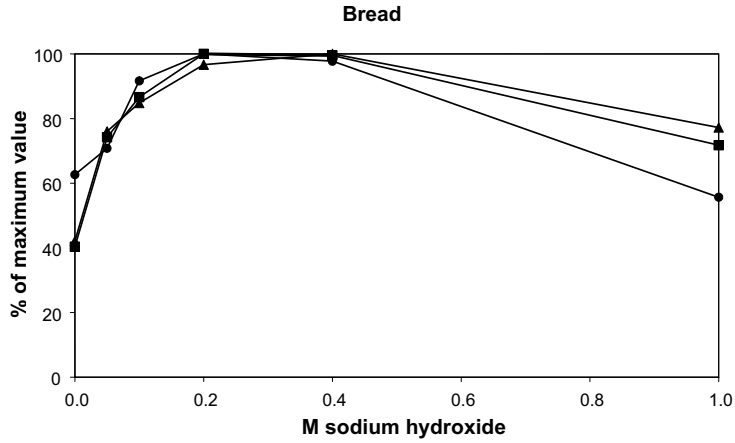

FIGURE 2.3 Influence of sodium hydroxide concentration during alkaline extraction on yield of LARI (squares), PINO (triangles), and SECO (circles), from bread, expressed as percentage of the maximum yield.

from the analytical method decreased the lignan yield up to 80% (TABLE 2.1). For broccoli this was also the case, although to a smaller extent. For beverages extraction of lignans from the food matrix is not necessary. Therefore, a milder, separate alkaline hydrolysis was used for tea. This hydrolysis was performed at 20°C and 0.05 M sodium hydroxide. Omitting the alkaline hydrolysis did not affect the yield of LARI, PINO and SECO, but fourfold enhanced the yield of MAT. Thus, analysis of tea was further performed without alkaline hydrolysis.

Enzymatic hydrolysis

Type of enzyme. Four different enzyme preparations, almond β-glucosidase, Rapidase LIQ+, *Helix pomatia* β-glucuronidase/sulfatase and bovine liver glucuronidase were first tested on isolated SECO diglucoside (an alkyl β-glucoside) and PINO diglucoside (an aryl β-glucoside). Rapidase LIQ+ is a commer-

TABLE 2.2 Aglycone yield from PINO diglucoside and SECO diglucoside and stability of lignan aglycones during enzymatic hydrolysis conditions.

Enzyme	Hydrolysis conditions	Aglycone yield (%)*		Stability of lignan aglycones (%)†			
		PINO	SECO	LARI	PINO	SECO	MAT
Helix pomatia β-glucuronidase /sulfatase	500 units‡, 16 h, 37°C	94 ± 3	90 ± 5	97 ± 3	100 ± 7	93 ± 5	101 ± 5
Bovine liver β-glucuronidase	500 units‡, 16 h, 37°C	12 ± 2	0 ± 0	99 ± 3	99 ± 1	97 ± 2	100 ± 0
β-glucosidase from almonds	50 units§, 16 h, 37 °C	100 ± 10	13 ± 0	110 ± 13	105 ± 1	105 ± 12	102 ± 1
Rapidase LIQ+	200 µL, 3 h, 37°C	84 ± 8	100 ± 5	117 ± 0	98 ± 2	113 ± 3	99 ± 2

*Results are expressed as percentages of the maximum value; mean of two duplicate analyses ± SD. †Results are expressed as percentage of standards without enzyme incubated for 16 h at 37°C; means of duplicate analyses ± SD. ‡Units β-glucuronidase. §Units β-glucosidase.

cial enzyme preparation, used in the fruit juice industry. It has a broad range of enzyme activities, such as pectinase and cellulase, but no specified β-glucosidase activity. *Helix pomatia* is also not recognized as a β-glucosidase, but it has been used before to liberate lignans,[31,32] and isoflavone aglycones.[36] The amount and hydrolysis conditions for each enzyme (TABLE 2.2) were based on enzyme specifications from the manufacturers or previous optimization with test products (results not shown).

Rapidase LIQ+ and *Helix pomatia* β-glucuronidase/sulfatase were able to convert both PINO diglucoside and SECO diglucoside to their aglycones (TABLE 2.2). β-Glucosidase yielded the highest amount of PINO aglycones, but only 13% of SECO aglycones compared to LIQ+. β-glucuronidase from bovine liver yielded no SECO aglycones and only 11% of PINO aglycones compared to the maximum value obtained with β-glucosidase. Broad unspecified enzyme preparations also have the potential to break down lignan aglycones. Therefore their stability during enzymatic hydrolysis was tested. Recovery was compared to a standard incubated without any enzyme added. There was no lignan breakdown due to the different enzymes (recovery of aglycones 93-117% compared to the standard without enzyme) (TABLE 2.2). The presence of lignans in the enzyme preparations was evaluated by incubation of the enzyme with sodium acetate buffer. None of the enzymes contained lignans except for β-glucosidase (from almonds) and Rapidase LIQ+, which contained traces of LARI.

Thus, both Rapidase LIQ+ and *Helix pomatia* were suitable for the hydrolysis of both types of glucosides. *Helix pomatia* β-glucuronidase/sulfatase was

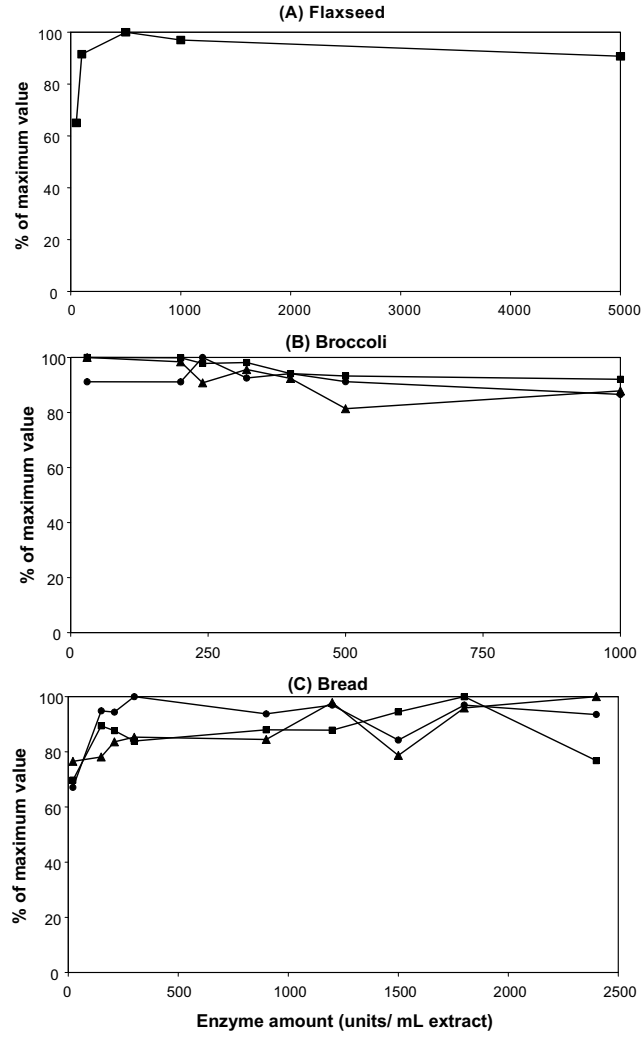

FIGURE 2.4 Influence of enzyme amount (β-glucuronidase units/mL extract) on yield of LARI (squares), PINO (triangles), and SECO (circles) from (A) flaxseed, (B) broccoli and (C) bread, expressed as a percentage of the maximum yield.

chosen for further use, since this is standardized, easily available for use in a laboratory environment, and contained no lignans.

Amount of enzyme. For the selected enzyme (*Helix pomatia)* the amount required to hydrolyze all glycosides was determined for flaxseed, broccoli, and bread (FIGURE 2.4). If possible, extracts were already diluted before the enzymatic hydrolysis, in order to avoid signal suppression during MS detec-

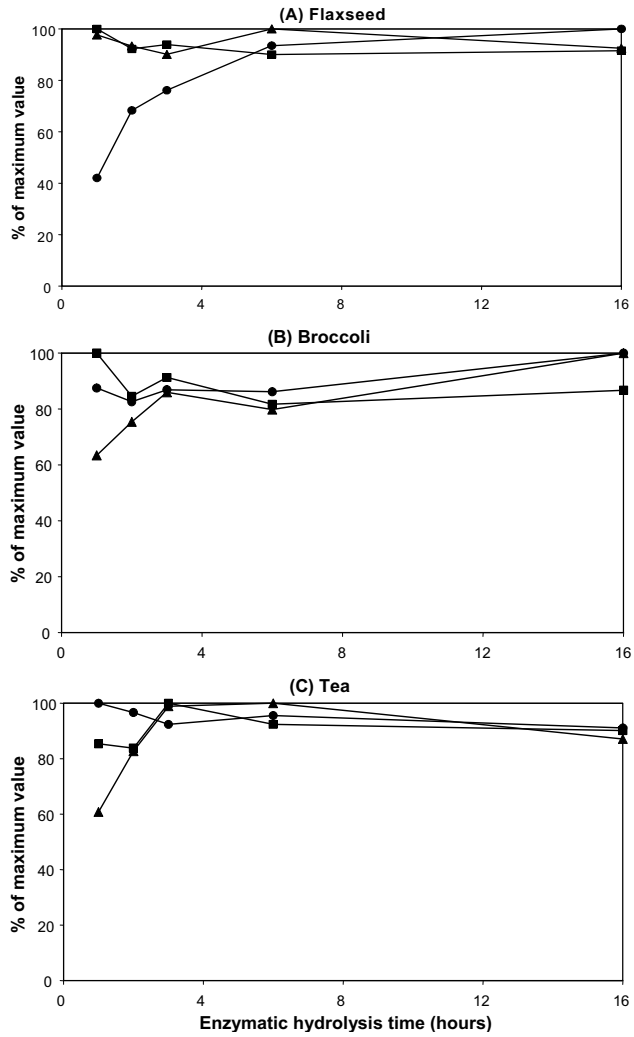

FIGURE 2.5 Influence of enzymatic hydrolysis time on yield of LARI (squares), PINO (triangles), and SECO (circles) from (A) flaxseed, (B) broccoli and (C) tea, expressed as a percentage of the maximum yield.

tion. Flaxseed was diluted 10×, and broccoli was diluted 5×. Bread was analyzed undiluted. For flaxseed, the optimal yield of SECO was achieved at 500 units β-glucuronidase per mL extract. Only the results of SECO are shown, because it is present in high amounts compared to the other lignans, which already had a maximum yield at the lowest enzyme amount. Within the tested range, the influence of the enzyme amount was modest for bread and broccoli. No substantial increases in aglycone yield were observed after 500

units per mL extract. Thus 500 units β-glucuronidase activity, corresponding to 0.83 mg *Helix pomatia* per mL extract, was chosen for the final method.

Hydrolysis time. Subsequently the effect of the hydrolysis time was evaluated (FIGURE 2.5). For broccoli and tea yield of LARI, PINO, and SECO did not substantially change after three hours, but for flaxseed there was still a relatively large increase in SECO between three and six hours. Sixteen hours of enzymatic hydrolysis was chosen for all products to ensure that optimum lignan yield was reached.

Chromtatographic and detection conditions

First we pursued a method with electrochemical detection. This was performed with a coulometric detector (Coularray detector, ESA Chelmsford, MA, USA) set at 300, 350, 650 and 800 mV. However, the specificity proved not to be adequate resulting in quite a number of interfering peaks. We tried several HPLC columns (with higher resolution than we used for MS detection), combined with several gradients and modifiers and/or simple cleanup procedures, but this did not improve the specificity enough to enable quantification of lignans in our test samples. Our aim was to develop a method with relatively simple sample preparation, and we thus concluded that this was not feasible with electrochemical detection. Therefore MS was chosen for the detection of the lignans. Both electrospray and APCI in the negative and positive mode were tested by infusion of pure enterolactone. Since APCI in the negative mode gave the highest sensitivity for enterolactone, this was also selected for quantification of the plant lignans. Cone voltages and collision energies were optimized using infusion of pure standards of the plant lignans. The most abundant product ions were chosen for multiple reaction monitoring (MRM) transitions. FIGURE 2.2 shows chromatograms of the selected transitions for a typical analysis of tea. Addition of ammonium acetate to the mobile phase largely decreased the sensitivity (especially for PINO) so only water and methanol were used as mobile phases. This did not influence the stability of the retention times. The desolvation gas flow was optimized by injection of standards of 200 ng/mL. Decreasing the desolvation gas flow from 500 to 100 L/h increased the sensitivity, signal to noise (S/N) ratios and signal stability.

Method validation

Calibration and quantification. In most cases, the slope of the calibration lines in food matrix was larger than the slope of the calibration lines prepared in

TABLE 2.3 Detection limits for lignans, as pure standards, in solid foods and beverages.*

Compound	Pure standards (ng/mL)	Solid foods (μg/100 g dw)†	Beverages (μg/100 mL)
LARI	1.6 ± 0.2	4 ± 0.5	0.2 ± 0.02
PINO	3.6 ± 0.1	10 ± 0.4	0.4 ± 0.01
SECO	2.3 ± 0.5	6 ± 1.4	0.2 ± 0.06
MAT	2.4 ± 0.5	6 ± 1.2	0.3 ± 0.05

*Mean of five triplicate measurements ± SD. †Dry weight, products were freeze-dried and milled before the analysis.

TABLE 2.4 Recovery (%) of lignans added to bread and tea.*

	Bread (n = 5)†		Tea (n = 4)‡	
Compound	100% addition	200% addition	50% addition	100% addition
LARI	123 ± 16	115 ± 18	118 ± 28	108 ± 33
PINO	110 ± 6	119 ± 9	112 ± 25	106 ± 37
SECO	82 ± 21	74 ± 6	73 ± 18	77 ± 28
MAT§	55 ± 5	51 ± 5	99 ± 11	102 ± 16

*Lignan standards were added at approximately 100 and 200% of the value in bread, and 50 and 100% of the value in tea. †Mean of five duplicate analyses ± SD. ‡Mean of four duplicate analyses ± SD. §Recoveries were calculated by adding two well detectable amounts.

aqueous methanol, indicating that the control for matrix effects was not optimal. Especially for bread the increases were relatively large (up to 28%). Bread extracts were concentrated 3.3× after the alkaline extraction (methanol was evaporated from 1 mL extract, until the residual volume was 0.3 mL) to increase the sensitivity of the method. However, concentration also increases the amount of food matrix in the extracts. Indeed the deviations increased when the bread extracts were more concentrated, especially for LARI and PINO. This can be explained by the fact that no deuterated standards for LARI and PINO were available, and corrections are based on d_8-SECO and d_6-MAT. To further investigate this matrix effect, calibration lines were constructed in 3.3× concentrated, undiluted or two times diluted bread extracts. Indeed, deviations could be decreased by dilution of the bread extract. For two times diluted bread extract, the maximum deviation of the slope was 11% (for LARI).

Limits of detection. The limits of detection ranged from 1.6 (LARI) to 3.6 (PINO) ng/mL (TABLE 2.3) corresponding to 4-10 μg/100 g dry weight for solid foods, and 0.2-0.4 μg/100 mL for beverages.

Recovery. The recoveries of lignans added to bread and tea are presented in TABLE 2.4. The recoveries of LARI and PINO were relatively high (106-123%).

The recovery of SECO ranged from 73-82%. For MAT recovery from bread was low (51-55%), but from tea it was close to 100% (99-102%).

Precision. The within-run CV was calculated by averaging the CVs of duplicate analyses at 6 (broccoli), 7 (bread) or 4 (tea) separate days. The within-run CV ranged from 6.0% (PINO in tea) to 20.8% (SECO in tea). The between-run variability was calculated after averaging the duplicate values from each day. The between-run CV ranged from 6.2% (LARI in broccoli) to 32.5% (SECO in bread).

Stability. No significant differences were found between sample extracts analyzed directly and extracts analyzed after 9 days storage at –80°C.

Discussion

In this study we have optimized a quantitative LC-MS method for the four major enterolignan precursors LARI, PINO, SECO and MAT. Previous methods were developed only for the quantification of SECO and MAT, or for specific lignan-rich foods such as flaxseed.

Combining the methanolic extraction with alkaline hydrolysis proved to be an important step to increase the lignan yield. The largest effect was found for the extraction of SECO from flaxseed. When alkaline hydrolysis was omitted the lignan yield decreased by 81%. This might be explained by the fact that SECO diglucoside in flaxseed forms oligomers with 3-hydroxy-3-methylglutaric acid via ester bonds.[37] These bonds are cleaved during the alkaline hydrolysis, thus releasing lignan glycosides from the oligomer. For bread, omitting the alkaline hydrolysis decreased the yield by 45-61%, for broccoli the decrease was 2-22%. Possibly, lignan oligomers are also present in other products, and methods with only enzymatic hydrolysis are likely to underestimate the lignan contents of such foods. Alkaline hydrolysis of tea did not influence the yield of LARI, PINO, and SECO, but largely decreased the yield of MAT. Probably lignans in tea are already free, so it is not necessary or even detrimental (for MAT) to use alkaline hydrolysis.

Enzymatic hydrolysis of isolated lignans showed that β-glucosidase from almonds did not efficiently hydrolyze SECO diglucoside (aglycone yield only 13% of the maximum value), which might explain the relatively low lignan values for methods that use only β-glucosidase.[30] *Helix pomatia* is not recognized as a β-glucosidase, but has been widely used to hydrolyze lignan[31,32] and isoflavone glycosides,[36] and we showed that SECO diglucoside and PINO diglucoside were efficiently hydrolyzed. However β-glucuronidase from bovine liver did not hydrolyze SECO diglucoside, and only a small amount of PINO diglucoside. So it

is not clear whether β-glucuronidase or other enzyme activities from *Helix pomatia* are responsible for the hydrolysis of lignan glucosides.

The sensitivity of the method is adequate for the analysis of a variety of foods with a wide range of lignan levels. The limits of detection of our method, 4-10 μg/100 g dry weight for solid foods and 0.2-0.4 μg/100 mL for beverages, compare favorable with other published methods. Only slightly lower detection limits (2-3 μg/100 g) have been published for the measurement of SECO and MAT in foods using ID-GC-MS-SIM.[32] Similar detection limits as we found for fluids, have been reported by Nurmi and co-workers for coulometric electrode array detection of lignans in wine (0.24-1.2 μg/100 mL).[33] For coulometric detection of MAT in flaxseed a higher detection limit of approximately 30 μg/100 g was found by Kraushofer and Sontag.[28] Horn-Ross and co-workers also reported a higher limit of detection of 25 μg/100 g food for HPLC-APCI-MS. [30]

The relatively high between- and within-run variation is the main limitation of the current method; however this is not uncommon for quantification relatively close to the detection limits. The variation is largely determined by the matrix of the various products. Broccoli has relatively high lignan contents and could be analyzed after dilution, resulting in within- and between-run CVs that were all less than 15%. For bread, a product with denser matrix and lignan concentrations closer to the detection limits, within-run CVs ranged from 10.1% to 14.7%, and between-run CVs from 17.8 to 32.5%. Tea lies more or less in between, with within-run CVs ranging from 6.0 to 20.8%, and between-run CVs of 9.2 to18.5%. Horn-Ross *et al.* published the only HPLC-APCI-MS method for the quantification of lignans in plant foods, but unfortunately did not document its variability.[30] For the analysis of lignans (including MAT) in plasma with HPLC-MS, within-run CVs of 6.1-19.7% were reported by Smeds and Hakala, for low to high concentration levels.[38] Mazur and co-workers reported within-run CVs of 4.6 to 6.6%, and between-run CVs of 8.2 to 14.6% for GC-MS analysis of SECO and MAT in foods.[32]

The slopes of the calibration lines nearly always increased when matrix was present compared to the calibration lines prepared in aqueous methanol. This was especially the case for LARI and PINO. It can be explained by the fact that no deuterated standards were available for those lignans, and d_8-SECO and d_6-MAT had to be used for their quantification. In order to minimize this error, it would be advisable to dilute samples as much as possible, but in practice this is not feasible since dilution also causes loss of sensitivity. Thus all samples were first analyzed undiluted. Only if lignan values in the sample extracts exceeded 1000 ng/mL, samples were reanalyzed with dilution of the alkaline extract. For undiluted sample, the maximum deviation of the calibration line was 26% for

LARI in bread, which would lead to an overestimation of the LARI content of 26%. However, by proper dilution overestimation can be reduced to 11%.

The recovery of LARI and PINO was relatively high (102-123%). This can be explained by the above mentioned matrix effects. For SECO the recovery was approximately 70-80%. For MAT added to tea, recovery was approximately 100%, but for bread recovery was low (51-55%). This is probably caused by limited stability of MAT aglycones under alkaline conditions. However omitting the alkaline hydrolysis resulted in an even lower yield (TABLE 2.1). Thus, the yield of MAT after alkaline extraction seems to be the result of release from the food matrix on one hand and breakdown of lignan aglycones on the other hand. We decided to retain the alkaline hydrolysis in the analytical method. This will lead to underestimation of MAT, but MAT is usually present in relatively low amounts in foods, compared to SECO[20] and the other enterolignan precursors (unpublished results). If labeled standards for all lignans become available, it would be advisable to add those standards at the beginning of the analytical procedure. This would enable correction of recovery losses, and decrease the problem of matrix interferences as well. We added deuterated standards just before the LC-MS analysis, because the stability of LARI and PINO during the analytical procedure differed from that of d_8-SECO and d_6-MAT (results not shown), and thus earlier addition would cause errors in correction.

In conclusion, combined methanolic extraction and alkaline hydrolysis followed by enzymatic hydrolysis with *Helix pomatia* β-glucuronidase/sulfatase, is an effective method for the release of lignan aglycones from different kind of plant foods. Combining the extraction with alkaline hydrolysis was an important step to increase the lignan yield, although it also causes breakdown of MAT. The LC-MS/MS method allows very specific and sensitive quantification of all four major enterolignan precursors, LARI, PINO, SECO and MAT. However, the accuracy and precision of the measurement of LARI and PINO could probably be improved if labeled standards for these compounds were also available. This would also allow correction of lignan losses during the analytical procedure. An advantage that this method offers compared to existing techniques is the relatively simple sample preparation. Since it requires no derivatisation, as for GC-MS, and specificity is improved compared to coulometric detection, extensive sample cleanup is not necessary.

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A database with lignan contents of Dutch plant foods

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3

Abstract:
Eterolignans (enterodiol and enterolactone) can potentially reduce the risk of certain cancers and cardiovascular diseases. Enterolignans are formed by the intestinal microflora after consumption of plant lignans. Until recently, only secoisolariciresinol (SECO) and matairesinol (MAT) were considered enterolignan precursors, but now several new precursors have been identified, of which lariciresinol (LARI) and pinoresinol (PINO) have a high degree of conversion. Quantitative data on the contents in foods of these new enterolignan precursors are not available. Thus, the aim of this study was to compile a lignan database including all four major enterolignan precursors. LC-MS was used to quantify LARI, PINO, SECO and MAT in 83 solid foods and 26 beverages commonly consumed in the Netherlands. The richest source of lignans was flaxseed (301 129 μg/100 g), which contained mainly SECO. Also, lignan concentrations in sesame seeds (29 331 μg/100 g, mainly PINO and LARI) were relatively high. For grain products, which are known to be important lignan sources, lignan concentrations ranged from 7 to 764 μg/100 g. However, many vegetables and fruits had similar concentrations, because of the contribution of LARI and PINO. *Brassica* vegetables contained unexpectedly high levels of lignans (185-2321 μg/100 g), mainly LARI and PINO. Lignan levels in beverages varied from 0 (cola) to 91 μg/100 mL (red wine). Only five of the 109 foods did not contain a measurable amount of lignans, and in most cases the amount of LARI and PINO was larger than that of SECO and MAT. Thus, available databases largely underestimate the amount of enterolignan precursors in foods.

Keywords:
lignans, phytoestrogens, food composition, secoisolariciresinol, matairesinol, pinoresinol, lariciresinol

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Introduction

Lignans are diphenolic compounds that are widely distributed in the plant kingdom. A large variety of plant lignans exist, but only a few of them are converted into the 'enterolignans' enterodiol and enterolactone by the intestinal microflora. Enterolignans are absorbed into the human body.

Until recently, only secoisolariciresinol (SECO) and matairesinol (MAT) were seen as enterolignan precursors, but new precursors have recently been identified, of which lariciresinol (LARI) and pinoresinol (PINO) have a high degree of conversion.[1] Lignans possess several biological activities, such as antioxidant and (anti)estrogenic properties and may thus reduce the risk of certain cancers as well as cardiovascular diseases.[2-5]

SECO and MAT have been quantified in a large number of plant foods. Flaxseed is the richest known source of these lignans. Other sources are grains, seeds, vegetables, fruits, and beverages.[6-9] These data have been incorporated into several phytoestrogen databases.[8,10,11] Until now, data on LARI and PINO contents of foods were not available.

A number of epidemiological studies on associations between enterolignan concentrations in biological fluids or intake of plant lignans and chronic disease risk has so far been conducted (reviewed by Arts & Hollman[5]). In case-control studies,[12-14] but not in prospective studies,[15-17] inverse associations have been found between plasma or urinary lignans and breast cancer risk For cardiovascular diseases, inverse associations with serum lignans were reported in two Finnish studies.[18,19] In addition, studies in which the relation between dietary intake of SECO and MAT and cancer risk was studied gave conflicting results. Protective associations were reported for breast,[20] ovarian,[21] endometrial,[22] and thyroid cancer.[23] In one study, a decreased risk of breast cancer was found only for high intakes of MAT, but not for SECO or SECO plus MAT.[24] In two other studies increased breast cancer risks were found with a high intake of SECO and MAT.[25,26]

To further evaluate the health effects of lignan intake, it is essential that the newly discovered enterolignan precursors are included. Thus, the aim of the present study was to compile a comprehensive database including all four major enterolignan precursors: LARI, PINO, SECO and MAT. Lignans were quantified in 109 plant foods commonly consumed in the Netherlands, including 83 solid foods and 26 beverages. Additionally, we studied the effect of cooking on the lignan content of a few vegetables.

Materials and Methods

Selection of foods

Plant foods were selected for analysis based on data from the Dutch National Food Consumption Survey conducted in 1997-1998. This survey was carried out among a sample of households representative of the Dutch population. Two-day dietary records were collected for 6250 persons aged 1-97 years.[27,28]

In general, plant foods with an average consumption of over 3 g per person per day were selected. Mixed dishes (*e.g.* apple pie, pizza) were not selected for analysis since their lignan content can be calculated from their ingredients using standard recipes. For fruits a limit of 1 g per person per day was used. Because of their relatively high consumption, a limit of 10 g per person per day was used for beverages. Waters and soft drinks (except cola) were disregarded. Apple juice was not analyzed because the lignan content of apples was very low (see below). Vegetable oils and fats with an average consumption of over 0.5 g per person per day were also added. Using this method, 42 plant foods were selected. Some important plant foods, for example leek and sweet pepper, were missed because they are ingredients of a large number of foods but are not consumed in large quantities as such. We therefore also used data on the consumption of primary agricultural products. These data were derived from the Food Consumption Survey data using the Conversion Model for Primary Agricultural Products developed at RIKILT.[29] Four extra fruits and eleven extra vegetables were selected, using the same criteria as before. In addition, foods that were high in lignans according to data in the literature but did not meet the aforementioned consumption criteria, were also selected (*e.g.* seeds, olives, and cashew nut). Tofu, soya milk, and legumes were added, since they might be important plant foods for specific population groups (*e.g.* vegetarians).

Sample collection

Foods were purchased in the form (fresh, canned, in jars, frozen) that was most commonly consumed according to the Food Consumption Survey. In order to enhance the representativeness of the food samples, products were bought at three different locations (fresh products) or from three major brands (processed foods) and composite samples were assembled. In addition, for fresh products, a minimum of 0.5 kg or three units was sampled at each location: an outlet of a nation-wide supermarket chain, a local grocery, and an open-air street market. The proportion in the composite samples reflected the sales of each food group at each location. For vegetables, the supermarket accounted for 78%, the grocery for 11% and the open-air mar-

ket for 11%, for fruits these values were 70, 12 and 18%, respectively. Potatoes were bought at two supermarkets (each contributing 45%) and a local grocery store (contributing 10%). Breads were also bought at two supermarkets (each contributing 37.5%) and a local bakery (contributing 25%). For processed foods, composite samples were assembled by mixing the three brands in equal proportions.

Sample preparation

Non-edible parts were removed from fruits and vegetables. Vegetables were not prepared further since cooking or frying was considered to have only a small effect on lignan content. This assumption was based on the fact that the first step in the lignan analysis consisted of alkaline extraction for one our at 60°C, which did not lead to considerable loss of lignans.[30] However, we checked this assumption, because during cooking and frying temperatures are higher and lignans might also be lost by leaching. A few vegetables were therefore analyzed both raw and prepared according to standard recipes. Lignans in potatoes, rice, and macaroni were determined in the prepared product according to standard recipes or the instructions on the packaging.

Composites of solid foods were either directly chopped under liquid nitrogen or cut into smaller pieces prior to freezing in liquid nitrogen, and stored at 20°C until freeze-drying was started within one month. After freeze-drying, samples were ground to a powder using a Tecator Knifetec 1095 sample mill (Rose Scientific Ltd, Edmonton, Alberta, Canada). Dry products, which did not require freeze-drying, such as seeds, muesli and wheat (meal and wholegrain), were ground before preparing the composite samples. Fruits were frozen in liquid nitrogen before grinding. All composites of solid foods were stored at –20°C until analysis.

Beverages were collected within 48 h of analysis. Composites were prepared immediately before analysis by mixing three major brands in equal proportions. Beer was degassed in an ultrasonic bath at room temperature. Since alcohol might influence the activity of the enzyme used in the analytical method, beer and wine were analyzed both with and without evaporation of alcohol (using a Zymark Turbo vap LV Evaporator, Zymark Corp., Hopkinton, MA) at 60°C, under a mild nitrogen flow (5-10 psi). Since the evaporation of alcohol produced no substantial changes in lignan concentrations, and increased the variation between duplicate samples, the lignan values of the analyses without evaporation of alcohol are presented here. Tea infusion was prepared by placing one tea bag (1 or 2 g) in boiling tap water (100 mL/g tea leaves). After 5 min, the tea bag was stirred a few times, after which it was removed. Coffee was prepared

according to Dutch custom in a coffee maker with paper filter: a volume of 275 mL of boiling water was dripped on 14 g of ground coffee. Chocolate milk was diluted ten times with sodium acetate buffer (0.05 M, pH 5.0), in order to avoid gelling during the ether extraction.

Standards

Pure standards of SECO (purity approximately 92%) and MAT (purity > 98%) were obtained from Plantech (Reading, England). LARI isolated from the wood of *Abies sachalinensis* and PINO isolated from *Euconmia ulmoides* oliv. bark were kindly provided by Dr. Ozawa (Rakuno Gakuen University, Japan), Dr. Deyama (Yomeishu Seizo Co., Ltd, Japan) and Dr. Nishibe (University of Hokkaido, Japan). Internal standards SECO-d_8 and MAT-d_6 were kindly provided by Dr. K. Wähälä (University of Helsinki, Finland).

Analytical method

The four major enterolignan precursors LARI, PINO, SECO and MAT were measured in each food sample using a liquid chromatography-tandem mass spectrometry (LC-MS) method previously described in detail.[30] In brief, lignans were extracted from solid foods with methanol-water (70/30 v/v) containing 0.3 M sodium hydroxide at 60°C for 1 h. After neutralizing and centrifuging, an aliquot of the supernatant was transferred to a test-tube, and methanol was evaporated under a mild nitrogen flow. The extract obtained, or pure beverage, was incubated overnight with *Helix pomatia* β-glucuronidase/sulfatase in sodium acetate buffer (0.05 M, pH 5.0) at 37°C. After enzymatic hydrolysis, samples were extracted twice with diethyl ether. The organic phases were combined, evaporated to dryness and dissolved in methanol/water (30% v/v). Internal deuterated standards were added, and samples were subjected to LC-MS analysis, with atmospheric pressure chemical ionization (APCI) in the negative-ion mode. Multiple reaction monitoring was performed with the following precursor/product combinations: LARI (359.1/329.1) and SECO (361.1/165.1) with internal standard SECO-d_8 (369.2/168.3), and PINO (357.1/151.2) and MAT (357.1/83.1) with internal standard MAT-d_6 (363.2/83.1).

Analytical quality control

Control samples were included at the beginning and end of each series of analyses. Single batches of freeze-dried and ground broccoli and wholegrain wheat bread were used as control samples for solid foods. These samples had been stored at –20°C. Black tea infusion was used as control sample for

beverages. One batch of tea leaves was prepared by passing black tea (from tea bags) through two sieves of 0.8 and 0.355 mm. The middle fraction was stored at room temperature for use as control product. For each series of analyses, fresh tea infusion was prepared by adding 100 mL of boiling tap water to 1 g tea leaves. After 5 min this was stirred, and 50 mL was filtered through a 1.2 μm Acrodisc filter (Gelman Sciences, Ann Arbor, MI, USA).

Calibration standards of 20, 100, 200, 600 and 1000 ng/mL of each lignan, with 200 ng/mL of deuterated standards, were injected three times, in the beginning, middle, and at the end of each series of analyses. If the lignan values in the sample extracts exceeded 1000 ng/mL, the sample was reanalyzed after diluting the alkaline extract. For solid foods, the limit of detection (signal to noise ratio of 3) for LARI was 4 μg/100 g dry weight, for PINO 10 μg/100 g dry weight, and for SECO and MAT 6 μg/100 g dry weight. Depending on the moisture content of the food this corresponded to 0.2-10 μg/100 g fresh weight. Detection limits for beverages were for LARI and MAT 0.2 μg/100 mL, for PINO 0.4 μg/100 mL, and for MAT 0.3 μg/100 mL. The within-run reproducibility for the analysis of the control samples was 6-21%, and the between-run reproducibility 6-33%.[30] All samples were analyzed in duplicate. If differences between duplicates were more then 20%, the analysis was repeated and the mean values of the two duplicate analyses were used.

Results and Discussion

We found that almost all of the selected plant foods contained lignans (TABLES 3.1 and 3.2). Only five out of the 109 products did not contain a measurable amount of lignans. The amount of lignans in plant foods varied widely, from 0 to 301 000 μg/100 g fresh weight. Interestingly, in almost all products the newly discovered enterolignan precursors LARI and PINO were found in higher concentrations than the well-known precursors SECO and MAT. Thus, databases with only SECO and MAT largely underestimate the amount of enterolignan precursors. Besides flaxseed, grain products have been the main focus of lignan research. However, our results show that lignan contents of many of the vegetables and fruits are similar to those of the grain products when LARI and PINO are also included.

Any comparison of food composition data from various sources is complicated by geographical differences in foods, such as plant variety, growth conditions, and food processing. Additional differences may result from variations in ripeness or season of collection of the sampled foods. Keeping this in mind, we compared our results primarily with lignan data reported by Mazur *et al.*[6,7] and Horn-Ross *et al.*,[8] since these were the most comprehensive sources.

TABLE 3.1 Lignan content (μg/100 g fresh edible weight) of solid foods.*

Product (*scientific name*)	Type/processing	Moisture content (%)†	LARI	PINO	SECO	MAT	Total
OILSEEDS AND NUTS							
Flaxseed (*Linum usitatissimum* L)		-‡	3041	3324	294210	553	301129
Sesame seed (*Sesanum Indicum* L)		-	9470	29331	66	481	39348
Sunflower seed (*Helianthus annuus* L)		-	671	167	53	0	891
Cashew (*Anacardium occidentale* L)		-	496	0§	133	0	629
Peanut (*Arachis hypogaea* L)		-	41	0	53	0	94
Poppy seed (*Papaver somniferum* L)		-	10	0	0	0	10
GRAIN PRODUCTS							
Breads							
Wholegrain flaxseed bread		34	220	383	11845	26	12474
Multigrain bread		34	185	377	6163	19	6744
Rye bread	Dark	46	122	172	13	14	320
	Light	43	111	163	16	12	301
Wheat bread	Wholegrain	41	73	33	15	0	121
	Refined	37	38	28	17	0	83
	White	36	11	7	0	0	18
Currant/raisin bread		29	79	9	9	7	104
Other grain products							
Muesli (granola)#	Jordans, crunchy	-	250	497	17	0	764
	Albert Heijn, basic	-	120	210	13	0	343
	Edah, crunchy	-	63	129	17	0	210
Wheat (*Triticum aestivum* L)	Wholemeal	-	140	38	31	0	210
	White flour	-	18	9	0	0	27
Rice (*Oryza sativa* L)	Wholegrain, boiled	67	28	7	3	2	40
	White, boiled	67	7	0	0	0	7
Macaroni	White, boiled	68	7	5	4	0	15
VEGETABLES AND LEGUMES							
Brassica vegetables (*Brassica oleracea* L)							
Curly kale (cv. *acephala* DC Alef.)		84	599	1691	19	12	2321
Broccoli (cv. *botyris* L var. *italica* Plenk.)		87	972	315	38	0	1325
White cabbage (cv. *capitale* L Alef. var. *alba* DC)		89	212	568	8	0	787
Brussels sprouts (cv. *oleracea* L var. *gemnifera* DC)		83	493	220	34	0	747
Sauerkraut (cv. *capitale* L Alef. var. *alba* DC)		91	116	133	67	0	316
Red cabbage (cv. *capitale* L Alef. var. *rubra* DC)		91	178	90	9	0	276
Cauliflower (cv. *botrytis* L Alef. var. *botyris* L)		92	124	58	4	0	185
Allium vegetables							
Garlic (*Allium sativum* L)		62	286	200	50	0	536
Leek (*Allium porrum* L var. *porrum*)		89	37	3	38	0	78
Onion (*Allium cepa* L var. *cepa*)		90	19	0	18	0	36
Other vegetables							
French bean (*Phaseolus vulgaris* L ssp. *vulgaris*)		89	220	24	29	0	273
Sweet pepper (*Capsicum annuum* L)	Green	94	164	1	7	0	172
	Red	91	106	1	7	0	113
Carrot (*Daucus carota* L)		92	60	19	93	0	171
Courgette (*Cucurbita pepo* L var. *melopepo*)		94	64	37	18	0	119
Spinach (*Spinacea oleracea* L)	Frozen	94	68	12	2	0	82
Cucumber (*Cucumus sativus* L spp. *sativus*)		96	59	1	8	0	67
Tomato (*Lycopersicum esculentum* Mill.)		94	42	14	2	0	58
Chicory (*Chicorum intybus* L cv. *foliosum*)		95	6	25	17	0	48
Endive (*Chicorum endivia* L)		93	15	9	14	0	38
Pea (*Pisum sativum* L)	In jars	81	14	20	0	0	34
Potato (*Solanum tuberosum* L)	Nicola, boiled	80	17	0	2	0	20

TABLE 3.1 Continued

Product (*scientific name*)	Type/processing	Moisture content (%)†	LARI	PINO	SECO	MAT	Total
	Redstar, boiled	76	8	0	1	0	10
Lettuce (*Lactuca sativa* L)		96	5	4	8	0	16
Iceberg lettuce (*Lactuca sativa* L cv. *capitata* L)		95	2	0	9	0	11
Sweet corn (*Zea mays* L)	In jars	76	2	0	5	0	7
Beetroot (*Beta vulgaris* L var. *conditiva* Alef.)	Boiled	91	3	0	1	0	3
Mushroom (*Agaricus campestris* Fr.)		93	0	0	0	0	0
Legumes							
Baked beans in tomato sauce (*Phaseolus vulgaris* L)	In jars	71	21	9	8	0	37
Brown beans (*Phaseolus vulgaris* L)	In jars	69	13	3	10	0	26
FRUITS							
Apricot (A*rmeniaca vulgaris* L)		86	105	314	31	0	450
Strawberry (*Fragaria* × *ananassa* Duch.)		91	117	212	5	0	334
Peach (*Prunus persica* L Batch)		89	80	186	27	0	293
Pear (*Pyrus communis* L)		84	155	34	4	0	193
Nectarine (*Prunus persica* L Batch)		89	41	131	18	0	190
Raisins (*Vitis Euvitis vinifera* L)	White	-	153	0	9	19	181
	Blue	-	118	0	8	18	144
Grapefruit (*Citrus paradisi* Macfad.)	Pink	88	95	45	9	2	152
Cherries (*Prunus avium* L)		79	41	100	6	0	147
Kiwi (*Actinidia chinensis* Planch.)		83	17	0	112	0	129
Plum (*Prunus domestica* L)		86	4	74	4	0	82
Mandarin (*Citrus reticulata* Blanco)		86	57	21	3	1	81
Olives (*Olea europaea* L)	Black	65	36	37	7	0	80
	Green	77	5	13	26	0	45
Orange (*Citrus sinensis* L Osbeck)		86	47	24	5	2	78
Melon (*Cucumis melo* L)	Galia	89	44	22	5	0	71
Grapes (*Vitis vinifera* L)	Blue	80	52	0	4	5	60
	White	83	25	4	10	3	42
Pineapple (*Ananas comosus* L Merr.)	Canned	85	3	5	7	5	20
Apple (*Malus domestica* Borkh.)	Elstar	85	1	0	0	0	1
	Jonagold	86	1	0	0	0	1
Banana (*Musa* × *paradisciaca* L)		75	0	0	0	0	0
VEGETABLE OILS AND FATS							
Olive oil	Extra-virgin	-	4	243	0	0	248
	Regular	-	5	101	0	0	106
Margarine		-	7	0	32	0	39
Soya oil		-	0	0	0	0	0
Sunflower oil		-	0	0	0	0	0
OTHER							
Tomato paste		70	107	70	9	0	187
Tofu		80	61	61	18	0	140
Cocoa	Powder	-	26	26	8	0	60
Chocolate	Plain	-	20	23	0	0	44

*Mean of duplicate analyses of composite samples. †Determined by freeze-drying. ‡Not freeze-dried. §Below detection limit: 4 ug/100 g dry weight for LARI, 10 μg/100 g dry weight for PINO, and 6 μg/100 g dry weight for SECO and MAT; corresponding to 0.2-10 μg/100 g fresh weight, depending on the moisture content. #Separate samples analysed instead of composite.

TABLE 3.2 Lignan content (μg/100 mL) of beverages.*

Product	Type/brand	LARI	PINO	SECO	MAT	Total
ALCOHOLIC BEVERAGES						
Wine						
Red wine†	Red, South Africa	15.9	6.3	61.3	7.8	91.3
	Red, France	16.1	9.5	47.5	5.9	78.9
	Red, France	8.6	11.9	41.7	6.9	69.1
White wine†	White, France	11.9	3.0	7.6	3.0	25.5
	White, Germany	7.3	1.7	12.2	2.7	23.8
	White, South Africa	4.6	2.5	5.2	3.1	15.5
Beer						
Beer (lager)	Grolsh	9.2	22.2	0.8	0.0‡	32.2
	Heineken	9.0	21.7	1.0	0.0	31.6
	Bavaria	5.9	12.6	0.0	0.0	18.5
NON-ALCOHOLIC BEVERAGES						
Tea						
Black tea	Ceylon†	30.4	40.6	5.0	1.1	77.1
	English blend	30.8	33.6	5.4	1.4	71.2
	Earl Grey†	28.9	27.0	6.2	1.5	63.6
Green tea†	(with lemon flavor)	18.7	5.7	12.9	2.0	39.2
Coffee						
Coffee†	Albert Heijn, Perla Robusta	13.1	1.5	16.1	0.7	31.3
	Kanis & Gunnink	9.1	1.3	9.2	0.0	19.6
	Douwe Egberts	9.0	0.4	9.4	0.0	18.7
Juices						
Grape juice	Blue	6.5	3.7	10.8	3.9	24.8
Grape juice	White	3.3	0.7	2.5	1.0	7.4
Tomato juice		9.7	9.9	1.6	0.0	21.2
Orange juice	Regular	7.0	7.5	2.7	0.0	17.2
	With pulp	7.4	6.6	2.7	0.0	16.6
Grapefruit juice	Yellow	5.1	4.8	6.0	0.0	15.9
	Pink	5.0	3.3	6.7	0.0	15.0
Other						
Soya milk		6.6	30.0	1.1	0.0	37.7
Chocolate milk	Semi-skimmed	0.9	1.3	0.0	0.0	2.2
Cola		0.0	0.0	0.0	0.0	0.0

*Mean of duplicate analyses of composite samples. †Separate samples analyzed instead of composite. ‡Below detection limit: 0.2 μg/100 mL for LARI and SECO, 0.4 μg/100 mL for PINO, and 0.3 μg/100 mL for MAT.

Mazur and co-workers quantified SECO and MAT in a variety of Finnish foods with isotope dilution GC-MS,[31] and these data have been published in several original and review publications.[2,6,7,32-34] However, as they report lignan val-

ues only on a dry weight basis, it is not possible to compare our lignan values directly with these results.

Recently, some additional lignan values were published on the occasion of the construction of a Finnish phytoestrogen database.[11] Since these data were expressed on a fresh weight basis, we used these data in preference for comparison. In general, our results for SECO and MAT are in agreement with or slightly lower than those published by Mazur *et al.*, although larger differences exist for a few products, which will be discussed below. Horn-Ross and co-workers measured SECO and MAT in foods from California.[8] They also used an HPLC-MS method for quantification. In general, our data for SECO are in agreement with or slightly higher than their results. However, since their limit of detection is relatively high (25 μg/100 g) compared with ours (0.2-10 μg/100 g), they found lignan concentrations to be below the detection limit in many products.

The lignan contents reported in this paper are measured after alkaline extraction from solid foods. Under alkaline conditions, ester-linked oligomers of SECO in flaxseed are hydrolyzed to give the lignan monomer.[35] Alkaline hydrolysis increased the lignan yield of flaxseed about five-fold, but lignan yields from broccoli and bread were also substantially increased.[30] Although we only tested these products, this indicates that ester-linked lignans also occur in other foods besides flaxseed. In addition, because we showed that our carefully optimized alkaline extraction method gave reproducible results,[30] we decided to use this method for our food analyses.

Ideally, the amount of lignans extracted by the analytical method should reflect the lignans that are available in the human body. So, can these ester-linked lignans be converted to enterolignans in the human body? Until now, no bioavailability studies have been performed that could answer this question. Andreasen *et al.* showed that human small intestine mucosa and colonic microflora contain esterase activity able to release diferrulic acids from diferrulate esters,[36] so it is likely that these ester-linked lignans have physiological relevance. Lapierre *et al.*[37] showed that 8-8'diferrulate is liberated from bran lignins under alkaline conditions similar to ours, implying that our alkaline extract might contain lignans that had been incorporated in lignin structures. A rat study indeed suggested that lignins from wheat and rye bran increased urinary excretion of enterolignans.[38] However, we think that this paper probably overestimated the contribution of these lignins to the enterolignans production. Thus, alkaline extraction seems to be able to account for physiological relevant lignans incorporated in lignins. Future human studies should, however, provide insight into the real potential of various kinds of bound lignans as enterolignan precursors.

The recovery of lignan aglycones added to control foods was regarded satisfactory (73-123%), except for MAT added to solid food (51-55%).[30] Thus, the concentrations of MAT in solid foods that we report here are somewhat underestimated. The low recovery of added MAT is caused by its instability during the alkaline extraction. However, the yield of lignans (including MAT) from control foods was largely increased by addition of sodium hydroxide during the extraction.[30] Thus, alkaline extraction favors the release of matrix-bound MAT, which more than compensates losses by degradation. Besides, our own and previous results show that the amount of MAT in foods is usually relatively low compared to the other enterolignan precursors. Thus, underestimation of MAT will have only a small effect on estimations of the total dietary lignan content. In general, our results for MAT are lower than those of Mazur,[7] but for some products we found higher amounts. Horn-Ross *et al.*[8] detected MAT in only three foods. We measured two of these foods, and found lower amounts of MAT in both.

Oilseeds and nuts

The richest source of lignans was flaxseed. Flaxseed contained mainly SECO (294 210 μg/100 g), but LARI, PINO and MAT were also present in substantial amounts (553-3324 μg/100 g). Most of the previously reported values for SECO were higher than we found: 369 900 μg/100 g dry weight by Mazur *et al.*,[7] 385 000-670 000 μg/100 g (converted from SECO diglucoside) by Johsson *et al.*,[39] 1 261 700 and 880 000 μg/100 g by Liggins *et al.*,[40] and 495 700-1 006 200 μg/100 g by Kraushofer & Sontag,[41] but lower values were also reported (81 700 μg/100 g) by Obermeyer *et al.*[42] In addition, the amount of MAT (553 μg/100 g) was relatively low compared with previously reported values (1087 μg/100 g dry weight),[7] (5860 and 9090 μg/100 g dry weight),[40] and (700-2850 μg/100 g).[43] Both PINO[44,45] and LARI[46] have previously been identified in flaxseed, but quantitative data were not reported.

The second highest lignan concentration was found in sesame seeds, but in this case, PINO was the main constituent (29 331 μg/100 g), and LARI was also relatively abundant (9470 μg/100 g). The presence of PINO in sesame seeds has previously been described.[47-49] It is a precursor of the sesame lignans piperitol, sesamin and sesamolin.[48,49] The total lignan concentrations in sunflower seeds (891 μg/100 g) and cashew nuts (629 μg/100 g) were also relatively high, although the concentrations of SECO were low compared to those reported by Mazur *et al.*[7] Mazur *et al.* detected some MAT (4 μg/100 g dry weight) in cashew nuts, which we did not find. In poppy seeds we detected only LARI (10 μg/100 g), whereas Mazur & Adlercreutz also reported the

presence of SECO (14 μg/100 g dry weight) and MAT (12 μg/100 g dry weight).[7]

Grain products

Both wholegrain flaxseed bread and multigrain bread had a high lignan content (12 500 and 6700 μg/100 g), which can be attributed to flaxseed present in these breads. For breads without flaxseed, the highest lignan concentration was found in rye bread, but the levels of SECO and MAT were approximately two to three-fold lower than reported for Finnish rye[50] and rye bread.[51] For wheat bread, wheat, and rice, the lignan content decreased with the level of refinement. This in agreement with previous findings (for rye) that lignans are mainly present in the short and bran of grain, which are removed during refining of grain products.[52]

Vegetables and legumes

Brassica vegetables (cabbages, Brussels sprouts, kale) contained unexpectedly high levels of lignans (185-2321 μg/100 g), mainly due to LARI and PINO. The amount of LARI plus PINO in *Brassica* vegetables was on average 45 times higher than that of SECO plus MAT. Our results for SECO in broccoli, cabbage and Brussels sprouts agree well with those of Mazur, but in broccoli and Brussels sprouts, they again report the presence of some MAT. Horn-Ross *et al.* reported a similar amount of SECO as we found in Brussels sprouts, but did not detect SECO or MAT in broccoli.[8]

A*llium* vegetables also contained substantial amounts of lignans (36-536 μg/100 g). The concentration of SECO in garlic reported by Horn-Ross *et al.* was approximately half of what we found, but they reported 38 μg/100 g MAT, which we did not detect in garlic. The amount of SECO in garlic reported by Mazur & Adlercreutz[7] was approximately twice as high as we found, and they reported only trace amounts of MAT. For leeks, the concentration of SECO reported by Valsta *et al.* was approximately three times lower than we found, and they also did not detect MAT.[11]

French beans (273 μg/100 g), sweet peppers (green: 172 μg/100 g, red: 113 μg/100 g), carrots 171 μg/100 g and courgettes (119 μg/100 g), also had relatively high lignan concentrations. For all other vegetables, total lignan concentrations were below 100 μg/100 g.

Fruits

Lignan values ranged from 0 for banana to 450 μg/100 g for apricot. Valsta *et al.* reported lignan contents of six fruits,[11] of which we also measured five.

The concentrations of SECO and MAT in pear, grapefruit, olive, and kiwi were somewhat higher than we found, whereas we found a higher amount of SECO in grapes. Horn-Ross *et al.* were able to detect SECO and MAT only in a few dried fruits (apricots, prunes and raisins), and in peaches.[8] The concentrations they reported for peaches agree well with ours.

Strawberries had a total lignan concentration of 334 μg/100 g. Mazur *et al.* found relatively high levels of SECO in berries.[53] Although we did not find a large amount of SECO in strawberry, the amounts of LARI and PINO were relatively large.

Raisins (white and blue) were one of the few products in which we detected MAT (19 and 18 μg/100 g, respectively). However, the amount of MAT in raisins was low compared with that of LARI (153 and 118 μg/100 g). Horn-Ross *et al.* reported a higher concentration of MAT in raisins (52 μg/100 g), and only trace amounts of SECO.[8]

The lignan concentration in apple was only 1 μg/100 g, which is interesting because apple is an important source of other polyphenols such as flavonoids.[54,55] In addition, Mazur & Adlercreutz[7] and Horn-Ross *et al.*[8] reported only trace amounts of lignans in apple.

Vegetable oils and fats

The amount of PINO in extra virgin olive oil (243 μg/100 g) was higher than that in regular olive oil (101 μg/100 g). This is in agreement with the data published by Owen *et al.*,[56] who found low levels of lignans in refined olive oil, but lignan levels of 65-10 000 μg/100 g in extra virgin oils. These lignans were identified as PINO, 1-acetoxy-PINO and 1-hydroxy-PINO. The amount of PINO that we found in extra virgin oil (of unknown origin), was much lower than that found in Spanish extra virgin olive oils (range 2000-4500 μg/100 g).[57] Possibly, (extra-virgin) olive oil in the Netherlands is more refined than in Spain. Margarine was also selected for analysis, since it contains vegetable fats. The lignan concentration in margarine was 39 μg/100 g; it contained mainly SECO. No lignans were found in soya- and sunflower oil.

Other solid foods

The concentration of lignans in tomato paste was higher than that of tomato, which can of course be explained by the concentration step that takes place during manufacturing. It also shows that lignans can (at least partially) survive the production process of tomato paste.

Soya products have primarily been included in phytoestrogen databases because of the high amount of isoflavones in soya, but soybeans were addi-

tionally shown to contain a relatively high amount of SECO (13-273 μg/100 g).[33] In tofu, however, we found only 18 μg/100 g. Horn-Ross[8] also detected a relatively high amount of SECO (140 μg/100 g) in soybeans, but did not detect any SECO or MAT in tofu. This is in agreement with our result, because of their high limit of detection of 25 μg/100 g.

Cocoa powder and plain chocolate contained a substantial amount of lignans, although the amount of SECO was approximately 50% lower than that reported by Valsta *et al.*[11] Similar to our findings, they did not detect any MAT.

Alcoholic beverages

Wine, especially red wine, is an important source of several polyphenols,[58] and we found that it also contained a relatively high concentration of lignans. Red wine contained on average 80 μg/100 mL, whereas white wine contained 22 μg/100 mL. SECO was the most abundant lignan in wine, except in one white wine in which LARI was more abundant. Our results for SECO and MAT were slightly lower than those reported by Mazur.[6] Nurmi *et al.* analyzed lignans in wines using HPLC with coulometric electrode array detection.[59] Besides SECO and MAT they also included other plant lignans. Their results for LARI, SECO, and MAT were slightly lower than ours. They could not exactly quantify PINO but reported that the amount was similar to that of LARI, which is also in agreement with our results.

On average, lager beer contained more lignans (27 μg/100 mL) than white wine, but less than red wine. Beer contained mainly LARI and PINO, and only a little SECO.

Non-alcoholic beverages

For the non-alcoholic beverages, the highest amount of lignans was found in tea, a product that is a rich source of various other polyphenols.[58] The amounts of lignans in the three blends of black tea that were analyzed were comparable (64-77 μg/100 mL), but the amount of lignans in green tea was lower (39 μg/100 mL). Mazur *et al.* expressed the lignan values of tea infusions on a mg/kg tea leaves basis.[32] If we convert our tea infusion values to mg/kg tea leaves, our results for SECO are approximately 1.5-2.5-fold lower than those of Mazur *et al.*, whereas the results for MAT are similar. For the preparation of their tea infusion, both the amount of tea leaves (per mL water) and the extraction time used by Mazur and co-workers were twice that of our method. Since we found lower values of SECO in both black and green tea, this might indicate that a higher proportion of SECO could be

extracted with longer extraction times. However, our aim was to analyze tea infusion as it is commonly consumed in the Netherlands.

The amount of lignans in coffee was lower than that in tea (19-31 μg/100 mL), and this is consistent with the results of Mazur *et al.*[32]

The total lignan concentration in juices was 7-25 μg/100 mL. As for red wine, and blue grapes, the amount of lignans was higher in blue grape juice than in white grape juice. In general, therefore, the lignan content of blue grapes seems higher than of white grapes, although the amount of lignans in white raisins was higher than in blue raisins. We did not find a difference between orange juice with and without pulp. We hypothesized that this could be caused by the fact that lignans were not released from the pulp. We thus also analyzed juice with pulp with the method for solid foods (including alkaline extraction). This resulted in slightly lower lignan concentrations compared to the analysis without alkaline extraction (results not shown). Thus, the pulp in orange juice did not contain a measurable amount of lignans.

Soya milk contained 38 μg lignans/100 mL, mainly LARI and PINO. We only measured 1 μg/100 mL of SECO in soya milk, whereas Adlercreutz *et al.*[60] found 10-20 μg/100 g in soya milk and drinks. Horn-Ross *et al.* reported 32 μg/100 mL SECO in soya milk.[8]

Effect of cooking of vegetables

Lignan values in boiled vegetables were on average 25% lower than those in raw vegetables (TABLE 3.3), whereas after frying, lignan concentrations were on average 30% higher. The increased lignan concentrations after frying can be explained mainly by the decreased moisture content of the fried foods. On a dry weight basis, the amount of lignans after frying decreased with 25%, comparable to what we saw for boiling. The vegetables were fried in margarine, which we have shown also to contain some lignans. However, we calculated that the maximum contribution from lignans in the margarine was less than 1%.

To our knowledge, effects of food preparation on lignan contents have only been reported for baking of bread, thermal treatments of olive oil, and roasting of pumpkin seeds. Muir & Westcott reported that SECO diglucoside (purified or as flaxseed) added to wheat flour before the preparation of bread, was stable in the bread making process.[61] Besides, they found that SECO diglucoside could withstand the higher temperatures in the core during baking. Brenes *et al.* found that microwave heating of olive oil for 10 min did not change the amount of lignans, including PINO.[62] Even after 25 h (simulated) frying at 180°C, only 20-50% of the lignans were lost, whereas other

TABLE 3.3 Comparison of lignan concentrations (μg/100 g) in raw and cooked vegetables.*

Product		Fresh weight LARI	PINO	SECO	Total	Prepared/ raw (%)†	Dry weight Total	Prepared/ raw (%)†
BOILED VEGETABLES								
Carrot	Raw	60	19	93	171		2039	
	Boiled	73	27	77	178	104	2058	101
Chicory	Raw	6	25	17	48		895	
	Boiled	3	12	13	29	60	567	63
Endive	Raw	15	9	14	38		528	
	Boiled	6	10	11	27	72	339	64
Potato (Nicola)	Raw	28	0‡	4	31		149	
	Boiled	17	0	2	20	63	98	65
FRIED VEGETABLES								
Onion	Raw	19	0	18	36		349	
	Fried	23	0	27	50	136	288	83
Sweet pepper (red)	Raw	106	1	7	113		1255	
	Fried	143	2	14	159	140	983	78
Sweet pepper (green)	Raw	164	1	7	172		2685	
	Fried	182	3	8	193	112	1747	65

*Mean of duplicate analyses. †Lignan concentration in boiled or fried vegetable compared to raw vegetable. ‡Below detection limit: 4 ug/100 g dry weight for LARI, 10 μg/100 g dry weight for PINO, and 6 μg/100 g dry weight for SECO.

phenolic compounds were almost completely destroyed. When olive oil was boiled with water (at pH 4-6) for 30 min, a large proportion of lignans leached into the water phase, but the total decrease in the lignan concentration was only 30%, irrespective of the pH. Murkovic *et al.* reported that SECO in pumpkin seeds was completely destroyed after 20 min of roasting.[63] Thus, a further evaluation of the effects of food processing might increase the reliability of lignan intake estimations.

In summary, almost all of the 109 measured products contained lignans, and in most cases the amount of LARI and PINO was larger than that of SECO and MAT. Thus, available databases largely underestimate the amount of enterolignan precursors in foods. The database reported here will enable a more comprehensive evaluation of the health effects of lignan intake.

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Lignan intake and major food sources of lignans in the Netherlands

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Abstract:
Enterolignans (enterolactone and enterodiol) are phytoestrogens that are formed by the colonic microflora from plant lignans. They potentially reduce the risk of certain types of cancer and cardiovascular diseases. Initially, only secoisolariciresinol (SECO) and matairesinol (MAT) were considered to be enterolignan precursors, but recently new precursors such as lariciresinol (LARI) and pinoresinol (PINO) were identified. We recently developed a lignan database including four major enterolignan precursors. We used this database to estimate lignan intake in a representative sample of Dutch men and women participating in the Dutch Food Consumption Survey, carried out in 1997-1998. Median total lignan intake among 4660 adults (19-97 year old) was 979 μg/day. Total lignan intake did not differ between men and women; thus, the lignan density of the diet was significantly ($P < 0.001$) higher in women than in men. Lignan intake was strongly skewed towards higher values (range 43 to 77 584 μg/day, mean 1241 μg/day). LARI and PINO contributed 75% to lignan intake, whereas SECO and MAT contributed only 25%. The major food sources of lignans were beverages (37%), vegetables (24%), nuts and seeds (14%), bread (9%) and fruits (7%). Lignan intake was significantly ($P < 0.001$) correlated with intake of dietary fiber ($r_s = 0.46$), folate ($r_s = 0.39$) and vitamin C ($r_s = 0.44$). Also older persons, nonsmokers, vegetarians, and persons with a low BMI, or a high socioeconomic status had higher lignan intakes than their counterparts. In brief, this study shows that the amount of enterolignan precursors in the diet has previously been largely underestimated.

Keywords:
lignans, diet, phytoestrogens, intake

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Introduction

Lignans are diphenolic compounds present in plant foods. Some plant lignans can be converted by intestinal bacteria into the 'enterolignans' enterolactone and enterodiol.[1,2] Enterolignans are present in human plasma and urine, and have been reported to possess biological activities, such as (anti-) estrogenic and antioxidant action, and therefore may reduce the risk of certain types of cancer as well as cardiovascular diseases.[3-6]

It has long been assumed that only secoisolariciresinol (SECO) and matairesinol (MAT) were converted into enterolignans. SECO and MAT are present in seeds, grains, vegetables and fruits, tea, coffee and wine.[7] Flaxseed contains by far the highest lignan concentration (of SECO) of any food for which data have been published. More recently other enterolignan precursors, *i.e.* lariciresinol (LARI), pinoresinol (PINO), arctigenin, 7-hydroxy-MAT, and syringaresinol were discovered.[8] In an *in vitro* experiment, LARI and PINO had a conversion degree of 100% and 55%, respectively, which is similar to, or even higher than the conversion degree of SECO and MAT. The conversion degree of the other new precursors was much lower (15% or less).

Observational studies (reviewed in reference 6) have used plasma or urinary enterolignans or lignan intake to estimate exposure, and both methods have given conflicting results. In case-control studies,[9-11] inverse associations between plasma or urinary lignans and breast cancer risk were observed, but in prospective studies, this was not confirmed.[12-14] For cardiovascular diseases, inverse associations with serum lignans were reported in two Finnish studies.[15,16] Studies regarding lignan intake and disease have only considered SECO and MAT. Protective associations have been reported for breast,[17,18] ovarian,[19] endometrial,[20] and thyroid cancer.[21] In one study, a protective effect for breast cancer was found only for high intakes of MAT, but not for SECO or the sum of SECO and MAT.[22] In a case-control study in a multiethnic population in the USA, no association was found between intake of SECO and MAT and breast cancer risk,[23] whereas in one cohort study, a high intake of SECO was associated with increased breast cancer risk.[24] However, this association was no longer statistically significant after adjustment for alcohol intake.[24]

To further evaluate the potentially protective effects of plant lignan intake, it is essential to include the newly discovered lignan precursors, LARI and PINO. Thus, we recently developed a method to determine LARI, PINO, SECO and MAT in foods,[25] and a food composition database with data on these major enterolignan precursors.[26] We used this database to estimate the intake of these lignans in a large, representative population sample in the

Netherlands. In addition, we identified the main food sources of lignans, and dietary and lifestyle factors associated with lignan intake.

Subject and Methods

Food consumption survey

As previously described in detail,[27] the National Food Consumption Survey 1997-1998 was carried out among a sample of households representative of the Dutch population.[28] A household was defined as one or more persons living together in one house, eating together a home-prepared hot meal for at least four days per week. Institutionalized persons, persons who did not speak Dutch sufficiently to complete the interview, and children younger than one year old were excluded. A total of 6250 persons (2885 men and 3365 women) aged 1-97 years, from 2564 different households participated in the study. The response rate was 68.5%. In the present study, we used only data of adults (≥19 year, n = 4661). Food consumption data were collected by trained dietitians using a two-day dietary record. The record days were equally distributed over the seven days of the week and over the year (holidays excluded). Intakes of nutrients were calculated using the 1996 release of the Dutch Food Composition Database.[29] Intake of folate was calculated using the 2001 release,[30] because data on folate had expanded since the earlier release. Other variables included age, height, weight, socioeconomic status (based on occupation and attained educational level) and lifestyle variables such as smoking and use of a vegetarian diet.

Lignan contents

The lignan database used comprises lignan contents of a comprehensive set of Dutch plant foods containing data on 83 solid foods, such as oilseeds, nuts, grain products, fruits, vegetables, and legumes, and 26 alcoholic and nonalcoholic beverages.[26] Plant foods were selected for inclusion in the database based on data from the Food Consumption Survey. In general, plant foods with a mean consumption of over 3 g per person per day were selected. For fruits a limit of 1 g per person per day was used, and for beverages this was 10 g per person per day. LARI, PINO, SECO and MAT were quantified using a liquid chromatography-tandem mass spectrometry method.[25] Lignans were measured in composite samples that existed of products bought at three locations: an outlet of a nation-wide supermarket chain, a local grocery, and an open-air street market (fresh products), or of three brands (pre-packaged products).

Lignan intake calculations

For each person, lignan intake was calculated by multiplying the consumption of each food with its lignan content. Food data were coded into 1167 entries of the Dutch Food Composition Table.[29] For 13% of the foods, lignan contents were derived from chemical analyses of the food concerned or calculated based on the dry weight content, 18% was calculated from recipes, and 16% was derived from a similar food (*e.g.* for port wine lignan contents of red wine were assigned). For the recipe calculations, we used standard recipes.[29,31] When standard recipes were not available, they were derived from food labels. For 35% of the foods, we assumed that there were no lignans present (mainly animal products). For the remaining 18% of the foods, a lignan content of zero was assigned. These were mainly products used by less than 1% of the population, since all plant foods with a relatively high consumption level were selected for analysis.[26]

Statistical analysis

Statistical analyses were performed using the SAS statistical package (SAS, release 8.02, SAS Institute, Cary, NC, USA). A P value of less than 0.05 was considered statistically significant. Children (<19 year, n = 1539) and pregnant women (n = 50) were excluded from the analyses. One person was excluded from the analyses, since he received enteral feeding only. Thus, the study population consisted of 4660 persons: 2116 men and 2544 women.

Mean and median lignan intake (μg/day), lignan density (μg/MJ/day), and the contribution of each individual lignan to the mean total lignan intake were calculated. Since previous studies have only considered intake of SECO and MAT, we have evaluated the agreement between quartiles of intake of the four lignans and quartiles of intake of SECO plus MAT using the percentage agreement (%) and weighted kappa statistic.[32] The contribution of individual foods and food groups to the mean lignan intake were calculated, and the percentage of users of each food or food group was determined. Mean lignan intakes for men and women, adjusted for age, and intakes in age categories adjusted for gender, were compared using analysis of covariance after log transformation of the individual lignan intakes. Similarly, mean lignan intakes stratified by other selected lifestyle characteristics were adjusted for age and gender. Spearman's partial rank-order correlation coefficients were calculated between total lignan intake, intake of selected nutrients, and intake of each of the individual lignans, after adjusting for total energy intake.

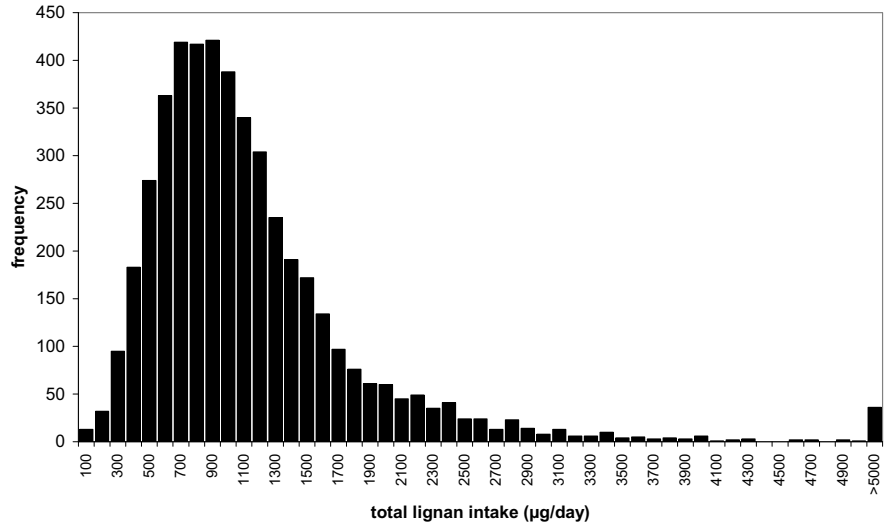

FIGURE 4.1 Distribution of total lignan intake among 4660 adults in the Dutch Food Consumption Survey 1997-1998.

TABLE 4.1 Lignan intake (µg/day) among 4660 adults in the Dutch National Food Consumption Survey 1997-1998.

	Percentiles				
Kind of Lignan	P10	P50	P90	Mean	% Contribution
LARI	238	465	897	535	43
PINO	108	306	764	403	32
SECO	93	167	277	292	24
MAT	2	9	22	11	1
Total	510	979	1907	1241	

Results

Lignan intake ranged from 43 to 77 584 µg/day, and was strongly skewed towards higher values (FIGURE 4.1). The median lignan intake in this population was 979 µg/day (mean 1241 µg/day, SD 2052 µg/day), on the two days surveyed (TABLE 4.1). In each person all four lignans contributed to lignan intake, except for MAT, for which intake was zero in 27 persons (0.6% of the population). On a population level, LARI contributed 43% to the lignan intake, PINO 32%, SECO 24%, and MAT only 1%. Intake of each of the individual lignans correlated significantly ($P < 0.001$) with total lignan intake. After adjustment for total energy intake, Spearman correlation coefficients (r_s) were 0.95 for LARI, and 0.90 for PINO, 0.52 for SECO, and 0.64 for MAT. When we compared classification of subjects in quartiles based on the intake of the four lignans, and quartiles based on intake of SECO and MAT, 42.3%

TABLE 4.2 Contribution (%) of food groups and individual foods to mean lignan intake among 4660 adults in the Dutch National Food Consumption Survey 1997-1998.*

	Users (%)	LARI	PINO	SECO	MAT	Total
BEVERAGES	100.0	34.9	40.4	35.3	73.2	37.1
Tea	69.4	19.1	28.1	6.3	42.1	19.2
Coffee	89.1	11.4	1.6	23.3	13.7	11.0
Beer (lager)	22.3	2.0	6.3	0.3	0.0	3.0
Fruit juice	33.9	1.4	3.1	1.0	0.0	1.8
Wine (red and white)	20.4	0.7	0.6	4.2	17.4	1.6
VEGETABLES	93.5	31.3	26.4	8.9	2.7	24.2
Brassica (cabbages)	34.8	18.2	22.1	2.1	2.7	15.5
Green beans	15.0	5.0	0.7	1.2	0.0	2.7
Carrot	14.9	1.2	0.6	2.5	0.0	1.3
Tomato	29.0	1.1	0.6	0.1	0.0	0.7
NUTS AND SEEDS	34.6	4.0	6.8	41.2	6.4	13.7
Flaxseed	0.6	0.2	0.3	39.4	1.8	9.5
Sesame seed	2.9	1.6	6.4	0.0	3.7	2.8
Sunflower seed†	4.4	0.5	0.1	0.1	0.0	0.3
BREAD	98.8	11.2	8.9	5.2	7.3	9.0
Wheat bread	96.7	9.3	7.1	4.7	0.0	7.4
Rye bread	5.5	0.4	0.9	0.1	2.7	0.5
Currant/raisin bread	14.2	0.9	0.1	0.2	3.7	0.5
FRUIT	75.3	7.8	7.9	2.4	6.4	6.6
Pear	8.3	2.4	0.7	0.1	0.0	1.3
Orange	5.7	0.9	2.1	0.1	0.0	1.1
Strawberry	16.0	1.4	0.9	0.2	2.7	1.0
Peach/Nectarine	3.7	0.4	1.4	0.3	0.0	0.7
Mandarin	16.8	1.0	0.5	0.1	0.9	0.6
Cake, cookies	77.4	1.7	2.1	1.2	2.7	1.7
Potatoes, French fries, chips	86.4	2.9	0.0	0.8	0.0	1.4
Mixed dishes‡	20.7	1.4	2.7	0.5	0.0	1.6
Grain products (without bread)	59.7	1.5	1.8	0.5	0.9	1.4
Soup	38.1	1.1	0.7	0.8	0.0	0.9
TOTAL		97.9	97.7	96.8	99.7	97.6

*Listed are food groups or individual products with a contribution to total lignan intake of ≥ 0.5%. †Contributed 0.3% to total lignan intake. ‡Includes dishes such as pizza, spring rolls, and canned spaghetti.

of the subjects were classified into the same quartile, for 40.1% the classification differed one quartile, and for 17.6% of the subjects classification differed two or three quartiles. The weighted kappa was 0.38.

The major sources of lignans were similar for men and women (results not shown). Beverages were the most important lignan source, and contributed 37% of the mean total lignan intake in this population (TABLE 4.2). Within this group tea and coffee had the highest contribution to lignan

TABLE 4.3 Lignan intake according to selected characteristics among 4660 adults in the Dutch National Food Consumption Survey 1997-1998.*

		n	µg/day	
Age† (y)	19-30	949	844	(814-875)#
	30-50	2036	971	(947-996)
	50-70	1181	1097	(1062-1134)
	≥70	494	1097	(1043-1154)
Gender‡	men	2116	995	(971-1020)
	women	2544	979	(957-1001)
Smoker§	no	3225	1028	(1008-1048)¥
	yes	1431	898	(872-925)
Alcohol consumer§	no	905	1002	(983-1020)
	yes	3748	922	(888-958)
Vegetarian/vegan§	no	4591	981	(965-997)¥
	yes	69	1510	(1319-1728)
Socioeconomic class§$	A (high)	586	1116	(1066-1169)#
	B1	1193	1077	(1043-1113)
	B2	1005	1002	(968-1038)
	C	1643	898	(873-923)
	D (low)	229	825	(765-889)
BMI§ (kg/m^2)	< 25	2599	1015	(992-1038)#
	25-30	1577	976	(949-1005)
	> 30	479	879	(835-926)

*Values are geometric means (95% CI). †Adjusted for gender. ‡Adjusted for age. §Adjusted for age and gender. $Classified according to occupation and attained education level. #$P < 0.001$ for linear trend, derived from linear regression. ¥$P < 0.001$ for difference between groups, derived from analysis of covariance.

intake. Almost all MAT was derived from beverages (73%). Besides tea and coffee, also wine was an important source of MAT (17% contribution). *Brassica* vegetables (cabbages, kale, Brussels sprouts) were important lignan sources, especially of LARI (18% contribution) and PINO (22% contribution). Nuts and seeds contributed 14% to lignan intake. This was mainly due to oilseeds (flax, sesame, and sunflower seeds), although these seeds were only used by a small part of the population. The highest lignan intakes in our population were found in persons who consumed oilseeds. Without the contribution of oilseeds the maximum lignan intake was approximately 5 mg per day, compared to 78 mg when the use of these oilseeds was included. Other important sources in this population were bread, mainly wheat bread, and fruit.

In this population, lignan intake increased with age (TABLE 4.3). There was no difference in total lignan intake between men and women. However, since women had a lower energy-intake, the lignan density of the diet (lig-

nan intake/MJ) was significantly ($P < 0.001$) higher in women than in men. After adjusting for age, the mean lignan density (95% CI) was 124 (121-127) μg/MJ/day in women, and 96 (93-98) μg/MJ/day in men. After adjusting for age and gender, lignan intake was significantly higher in nonsmokers than in smokers, in vegetarians than in nonvegetarians, in subjects of higher socioeconomic classes than of lower socioeconomic classes, and in subjects with lower BMI compared to higher BMI. There was no statistically significant difference of lignan intake between users and nonusers of alcohol. After adjustment for total energy intake, total lignan intake was significantly ($P < 0.001$) correlated with intake of fiber ($r_s = 0.46$), folate ($r_s = 0.39$) and vitamin C ($r_s = 0.44$). These correlations were similar for men and women (results not shown).

Discussion

To our knowledge this is the first study to describe the intake of four major enterolignan precursors. Previous studies only included data on the consumption of SECO and MAT. In this representative sample of the Dutch population, SECO and MAT contributed only 25% to the mean total lignan intake of 1241 μg/day, whereas LARI and PINO contributed 75%. Thus, previous studies have largely underestimated the amount of enterolignan precursors in the diet. Besides, intake of LARI ($r_s = 0.95$) and PINO ($r_s = 0.90$) correlated stronger with total lignan intake than intake of SECO ($r_s = 0.52$) and MAT ($r_s = 0.64$). The exact agreement between quartiles based on intake of the four lignans and quartiles of SECO plus MAT, was only 42.3%, reflected by a low weighted kappa of 0.38. Thus in epidemiological studies, classification of subjects based on the intake of the four lignans will differ substantially from classification based on SECO and MAT only.

Although several studies have reported intakes of SECO and MAT, it is difficult to compare these results across populations. Besides actual differences in the lignan content of the diet, differences may also result from differences in reporting of intake data, composition of the population, and especially from the lignan food composition data used. Our estimate of the mean daily intake of SECO plus MAT (303 μg/day) is higher than that previously reported by Horn-Ross and co-workers for women in the USA (175 μg/day) and (108 μg/day),[24,33] but lower than that reported by De Kleijn and co-workers (645 μg/day),[34] for another population of women in the USA. Our result is similar to the reported intake for men and women in Finland (434 μg/day).[35] Keinan-Boker *et al.* reported considerably higher (1110 μg/day) intakes of SECO plus MAT for Dutch middle-aged and elderly women than we found.[36]

This may partly be explained by the difference in study population, since we found that intake increased with age. However, the difference in lignan composition data is probably more important. For the calculation of lignan intake Keinan-Boker and co-workers used the same method as De Kleijn.[34] Seven category scores, instead of exact lignan contents, were used for the calculation of lignan intake. These scores were based on the highest reported value found in the literature. For some products, the content of SECO and MAT was derived from the amount of enterolignans produced after *in vitro* fermentation. This leads to an overestimation of the amount of SECO and MAT if the product also contains other enterolignan precursors. For the present study, all foods were analyzed in one laboratory. We collected data on all important lignan sources in the Netherlands, and values for all foods were obtained with the same validated method.[25]

An important consideration in interpreting the results from our study is that the dietary survey was not designed to investigate lignan consumption. As a result, consumption of seeds was not inquired in detail. For example, no distinction was made between wholegrain wheat bread and multigrain bread. However, retail data show that in 1998, 8% of purchased breads were multigrain.[37] Multigrain bread differs in composition between different manufacturers or brands, and almost all multigrain breads contain flaxseed and various other seeds. Thus, in general, the lignan content of multigrain bread is much higher than that of wholegrain wheat bread. In order to evaluate the potential underestimation of lignan intake caused by the lack of data on multigrain bread, we assumed that 8% of the bread reported as wholegrain wheat bread was actually multigrain bread. The lignan content of multigrain bread was previously determined in a composite sample of six commonly consumed multigrain breads.[26] Using these assumptions, median total lignan intake increased from 979 to 1132 μg/day, 16% higher than in our previous estimation (TABLE 4.1). However, since the composition of multigrain breads is highly variable, we choose not to include the lignan intake from multigrain bread in our estimation (TABLE 4.1). Since the share of multigrain bread has been increasing since 1998 (till 12% in 2001, the last year for which data were available),[37] an important recommendation for future research is to evaluate the intake of seeds from bread in more detail.

Another limitation of the present study is that effects of food processing have only partly been taken into account. In general, the lignan contents of foods as they were usually consumed were available from chemical analysis. For example, fresh fruits were analyzed, as they were usually consumed. Values for dried or canned foods were derived from values of fresh fruits,

allowing for differences in dry weight. Most vegetables were only analyzed raw, although in 1998 the mean consumption of boiled vegetables was higher than that of raw vegetables. This choice was made, since many vegetables are also consumed raw, and other preparation methods such as (stir-)frying and simmering are also common. Since we previously found that the lignan content of some boiled vegetables was decreased (with 25% on average),[26] this may have caused overestimation of the lignan intake from vegetables.

In this study, beverages, vegetables, seeds, bread, and fruits were the major sources of dietary lignans. These lignan sources were similar to those found in previous studies in the Netherlands, Finland, Germany, and the USA,[22,33,34,36,38] even though previous studies only studied intake of SECO and MAT. However, the relative importance of sources varied between the populations studied. The contribution of vegetables, especially *Brassica* vegetables to lignan intake in our study, was higher than that in previous studies. This can be explained by the relatively high amount of LARI and PINO in these vegetables. Previously reported contributions of coffee and tea to lignan intake varied largely from 0.2%[34] to 44%,[33] both for postmenopausal women in the USA. Such large variations between similar populations probably do not reflect real differences in consumption, but are a result of differences in lignan data used in these studies. In previous studies, lignan data for brewed coffee and tea were not available, and thus lignan contents were extrapolated from coffee powder and tea leaves, using different assumptions. In our study we used the lignan content of brewed coffee and tea, prepared according to Dutch habits, which greatly improved the reliability of the intake calculations.

As reported earlier,[14] intake of lignans was highly correlated with intake of dietary fiber (r_s = 0.46). Strong correlations have also been reported for intake of dietary fiber with enterolignan concentrations in blood or urine.[14,39,40] Dietary fiber has been proposed as a proxy for lignan intake.[14] Although we also found a strong correlation between lignan intake and dietary fiber, the fact that fiber-poor beverages were the most important sources of lignans does not support the use of dietary fiber as a proxy for lignan intake.

In conclusion, this study shows that including LARI and PINO largely improves the estimation of the intake of dietary enterolignan precursors. In this population, intake of LARI plus PINO was approximately three-fold higher than that of SECO plus MAT. Besides, intake of LARI and PINO had a higher correlation with total lignan intake than SECO and MAT. Thus, including LARI and PINO will reduce exposure misclassification to enterolignans in epi-

demiological studies, and may give more insight into the association between lignan intake and chronic diseases. Lignan intake was positively correlated with the intake of dietary fiber, folate, and vitamin C, and with determinants of healthy lifestyle, such as nonsmoking, use of vegetarian diet, high socio economic class, and low BMI; thus, these factors must be considered in epidemiological studies assessing the health effects of lignan intake.

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Relation between enterolignans in plasma and intake of plant lignans

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5

Abstracts:
Enterolignans are phytoestrogenic compounds derived from the conversion of dietary lignans by the intestinal microflora that may be protective against cardiovascular diseases and cancer. To evaluate the use of enterolignans as biomarkers of dietary lignan intake, we studied the relation between plasma and dietary lignans. We determined the dietary intake of four lignans: lariciresinol (LARI), pinoresinol (PINO), secoisolariciresinol (SECO), and matairesinol (MAT) using the EPIC food frequency questionnaire, and plasma enterodiol (END) and enterolactone (ENL) concentrations were determined by LC-MS/MS. The population consisted of 637 men and women, aged 19-75 y, participating in a case-control study on colorectal adenomas. Participants did not use antibiotics in the preceding calendar year. We found a modest association between lignan intake and plasma END ($r_s = 0.09$, $P = 0.03$) and ENL ($r_s = 0.18$, $P < 0.001$). The correlation of total lignan intake with plasma enterolignans was slightly stronger than that of only SECO plus MAT. The plasma concentrations of both END and ENL were associated with intake of dietary fiber and vegetable protein, but not with intake of other macronutrients. The relation between lignan intake and plasma END was modulated by age and previous use of antibiotics, whereas for ENL it was modulated by weight, current smoking, and frequency of defecation. However, even when we included these nondietary factors in the regression models the explained variance in plasma END and ENL remained low (2 and 13% respectively).

Keywords:
lignans, phytoestrogens, intake, biomarker, human

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Introduction

Lignans are biphenolic compounds in plant foods, which belong to the group of phytoestrogens. Wholegrain products, vegetables, fruits, nuts, seeds, and beverages such as tea, coffee, and wine are dietary sources of lignans.[1,2] Plant lignans can be converted by intestinal bacteria into so-called enterolignans: enterodiol (END) and enterolactone (ENL). For about two decades only secoisolariciresinol (SECO) and matairesinol (MAT) were known to be precursors of enterolignans, but recently it was shown that also pinoresinol (PINO) and lariciresinol (LARI) are efficiently converted into enterolignans.[3]

Enterolignans possess several biological activities, by which they may reduce the risk of cancer and cardiovascular diseases. Enterolignans have weak estrogen-like activity,[4] may inhibit enzymes such as aromatase and 5α-reductase, and stimulate the production of sex hormone-binding globulin (SHBG).[5] In addition, plant lignans, and to a lesser extent also enterolignans, have antioxidant activity.[6] In epidemiological studies, some evidence for protection of lignans against hormone-related cancers and cardiovascular diseases was found, but results were not consistent.[7] Enterolignan concentrations in biological fluids have been used as a biomarker for lignan intake in several of these studies.

In order to assess the relative validity of these exposure measures, a few studies have examined the correlation between plasma and dietary lignans. So far, these studies have found only weak to moderate associations (r_s = 0.08-0.19),[8-10] possibly because they only included two of the dietary lignan precursors (SECO plus MAT), and one of the metabolites (ENL). Horn-Ross *et al.* used urinary END plus ENL as a biomarker for lignan intake,[11] and found a similar correlation with intake of SECO and MAT (r_s = 0.17). In one study additional dietary lignans have been included, but lignan contents were only available for milk, bread, and cereal products and it did not improve the correlation with serum ENL compared to inclusion of only SECO plus MAT.[12]

We recently developed and validated methods to measure four dietary lignans in foods and two enterolignans in plasma.[13,14] To evaluate the use of enterolignans as biomarkers of dietary lignan intake, we have studied the relationship between plasma and dietary lignans in the POLIEP-study, a case-control study on colorectal adenomas. We included both plasma lignans END and ENL and four dietary lignans (LARI, PINO, SECO and MAT). In addition we identified other determinants of plasma lignans, which may modulate the relation between plasma and dietary lignans.

Materials and Methods

Population

Subjects included in the current study were men and women participating in the POLIEP-study, a case-control study designed to investigate gene-environment interactions and the risk of colorectal adenomas. Participants were recruited among patients undergoing endoscopy in ten clinics in the Netherlands between June 1997 and October 2002. The study design has previously been described in more detail.[15,16] Eligible subjects were Dutch speaking, of European origin, 18-75 years old at the time of endoscopy, and had no hereditary colorectal cancer syndromes (*i.e.* familial adenomatous polyposis, hereditary nonpolyposis colorectal cancer), chronic inflammatory bowel disease, history of colorectal cancer, (partial) bowel resection, or serious disabling morbidity. In the POLIEP-study cases were defined as those with at least one histologically confirmed colorectal adenoma. In controls, diagnosis of any type of adenomas was negative at the endoscopy, and no history of any type of adenomas existed (based on medical record). The medical ethical committees of all participating hospitals and of Wageningen University approved the study protocol and all participants provided written informed consent.

Plasma was available for 1385 of a total of 1477 subjects. Before the endoscopy, subjects fasted and received medication to clean the colon. Because enterolignans are produced by intestinal bacteria, plasma enterolignan values were expected to be lower than normal at the time of endoscopy. Therefore, we excluded 350 subjects whose blood samples were drawn on the same day an endoscopy was performed. Antibiotic use can decrease enterolignan concentrations for 3-12 months.[17] We excluded an additional 397 subjects because they had taken antibiotics in the calendar year of blood sampling or because data on antibiotic use were missing. One subject was excluded because information on body weight was missing, which made it impossible to calculate the lignan dose. After these exclusions, data for 637 subjects remained in the analyses, 331 cases with adenomatous polyps and 306 endoscopy controls who never had polyps. The population included 355 women aged 19-75 y with a mean weight of 70 ± 13 kg, and 288 men aged 21-75 y with a mean weight of 84 ± 12 kg.

Data collection

Participants were requested to complete self-administered questionnaires on diet, medical history, and lifestyle according to habits in the year preceding their last endoscopy. Habitual physical activity was estimated using a short questionnaire.[18]

Dietary assessment. We assessed dietary intake with the validated Dutch European Prospective Investigation into Cancer and Nutrition Food Frequency Questionnaire (EPIC FFQ).[19,20] This questionnaire enables estimation of the mean daily consumption of 178 food items in the preceding year. Consumption data were converted into energy and nutrient data using the Dutch Food Composition Table.[21] Lignan intake was estimated using a recently developed database including contents of LARI, PINO, SECO, and MAT of commonly consumed Dutch plant foods.[2]

Collection of plasma samples. Venous blood samples were taken into vacuum tubes containing EDTA. Samples were taken between 8:00 AM and 8:45 PM, on average 4 months after endoscopy. Subjects did not fast. Samples were transported to our laboratory at Wageningen University in a foam fridge at 4°C. Within 48 hours they were centrifuged at 1187 × g; 10 min at 4°C and then kept at –80°C until analysis.

Plasma assays

We determined plasma END and ENL using a validated isotope-dilution LC-MS/MS method.[14] In brief, $^{13}C_3$-abeled END and ENL were added to the samples, and samples were enzymatically hydrolyzed in order to release aglycones from glucuronide and sulfate conjugates. Samples were extracted twice with diethyl ether, dissolved in 40% methanol/water, ether evaporated, filtered, and injected into the LC-MS/MS system. The samples were analyzed in 20 runs over a 12-wk period. The between-assay coefficient of variation was 14% for END and 10% for ENL. The limit of detection was 0.15 nM for END and 0.55 nM for ENL. When a plasma value was below the detection limit, we assigned to that sample a value of 0.5 times the detection limit to enable log-transformation of skewed data.

Statistical analysis

Participants were classified according to tertiles of total plasma enterolignans. To test for differences in demographic and lifestyle characteristics between tertiles, we used ANOVA for normally distributed variables, Kruskal-Wallis test for skewed variables and the chi-square test for categorical variables.

Spearman rank order correlations were calculated for absolute lignan intake as well as lignan dose (lignan intake per kg body weight) with plasma enterolignan concentrations. We used linear regression to evaluate whether the correlations between lignan intake and plasma enterolignans differed between total, incident, or prevalent cases and controls.

To identify dietary and nondietary determinants of plasma enterolignans, we used several regression models. Because the distribution of dietary and plasma lignans was skewed, log-transformed data were used. This also accounts for the apparent exponential relationship between plasma and dietary lignans. First, total lignan intake, age, sex, weight, smoking, physical activity, use of antibiotics (ever), polyps (ever), indication for endoscopy, and frequency of defecation were examined as potential determinants of plasma enterolignans by fitting univariate models. Second we constructed a full model that included all these nondietary variables. Finally, to identify dietary determinants of plasma enterolignan concentrations, we used multivariate models including the nondietary determinants that were significant ($P < 0.05$) in the full model for END or ENL, and energy intake. Dietary lignans, fiber, major energy-providing nutrients, and major food sources of lignans were considered as potential dietary determinants of plasma enterolignan concentrations. We used one 1-SD change in intake of potential dietary determinants to estimate the associated change in plasma enterolignan concentrations.

Statistical analyses were performed using SAS software (version 9.1, SAS institute, Inc, Cary, NC). Values in the text are geometric means (95% CI) unless otherwise indicated.

Results

As expected, participants in the highest tertile for total plasma enterolignans had higher intakes of all four dietary lignans than participants with lower plasma enterolignan concentrations, although the difference was not significant for SECO (TABLE 5.1). Participants in the highest tertile of total plasma enterolignans were on average older, more likely to be female, weighed less, less likely to smoke, and more likely to have a low frequency of defecation than participants with lower plasma enterolignan concentrations.

The plasma concentrations in the total population were 1.4 (1.3-1.6) nM for END and 11.3 (10.2-12.5) nM for ENL (TABLE 5.2). Six percent (n = 39) of the samples was below the detection limit of 0.15 nM for END and 1% (n = 6) was below the detection limit for ENL of 0.55 nM. The total lignan intake was 989 (963-1016) μg/d.

The correlation of total lignan intake with plasma END ($r_s = 0.09$, $P = 0.03$) was weaker than that with plasma ENL ($r_s = 0.18$, $P < 0.001$) (TABLE 5.2). When lignan intake was expressed per kg body weight (lignan dose), the correlations with plasma enterolignans were stronger than those for absolute lignan intake. Most of the correlations of plasma END and ENL with intake

TABLE 5.1 Characteristics of the study population according to tertiles of total plasma enterolignans (n = 637).*

Tertile	1 (n = 212)	2 (n = 213)	3 (n = 212)	*P*†
PLASMA ENTEROLIGNANS (nM)				
Total	5.0 (3.2-7.1)	15.5 (12.3-19.8)	40.6 (30.2-58.6)	
END	0.7 (0.4-1.2)	1.4 (0.8-2.7)	2.8 (1.7-5.1)	
ENL	3.8 (1.5-5.7)	13.8 (10.1-17.5)	37.3 (27.3-55.6)	
LIGNAN INTAKE (μg/d)				
Total	937 (740-1159)	1006 (798-1260)	1072 (873-1319)	<0.001
LARI	445 (348-542)	481 (377-577)	500 (406-625)	<0.001
PINO	303 (235-432)	334 (243-450)	377 (285-476)	<0.001
SECO	181 (139-222)	187 (150-211)	189 (154-232)	0.17
MAT	6 (3-11)	8 (4-11)	9 (5-13)	<0.001
GENERAL				
Age (y)	54 ± 13	57 ± 12	57 ± 12	0.01
Female (%)	54	51	63	0.04
BMI (kg/m^2)	26.8	25.8	24.9	<0.001
Weight (kg)	79 ± 16	77 ± 15	73 ± 13	<0.001
Current smoking (%)	30	27	16	0.02
Low physical activity (%)	32	38	26	0.14
BOWEL				
Polyps cases (%)	56	49	51	0.36
Indication for endoscopy (%)				
Complaints	65	62	62	0.15
Screening	26	33	32	
Other/unknown	10	5	7	
Frequency of defecation (%, n/wk)				
>7	53	44	26	<0.001
5-7	37	43	54	
1-5	9	11	17	
<1	1	2	3	
Diet change (%)§	24	30	31	0.22
MEDICATION				
Ever used antibiotics‡ (%)	78	77	74	0.57
Regular NSAID use (% ≥12 times/y)	26	36	30	0.30
Current oral contraceptive use (%)#	13	12	9	0.79

*Values are mean ± SD or median (25th percentile-75th percentile). †ANOVA for normal distributed variables, Kruskal-Wallis for skewed variables and chi-square test for categorical variables. ‡Persons who used antibiotics in the previous calendar year were excluded. #In women only, n = 355. §Diet change because of bowel complaints.

of the individual lignans were slightly weaker than those with total lignan intake, and intakes of SECO and MAT were not correlated with plasma END. The correlation between total lignan intake and plasma enterolignans did not

TABLE 5.2 Spearman correlation coefficients (r_s) between lignan intake and plasma lignan concentration in 637 men and women in the POLIEP-study.

			Plasma lignans (nM)*	
			END	ENL
			1.4 (1.3-1.6)	11.3 (10.2-12.5)
Lignan intake (µg/d)*				
LARI	463	(451-475)	0.09†	0.18§
PINO	328	(317-340)	0.10†	0.18§
SECO	180	(176-185)	0.02	0.10‡
MAT	6	(6-7)	0.07	0.21§
Total	989	(963-1016)	0.09†	0.18§
Lignan dose (µg/kg/d)*				
LARI	6.2	(6.0-6.4)	0.12‡	0.24§
PINO	4.4	(4.2-4.6)	0.13§	0.23§
SECO	2.4	(2.3-2.5)	0.06	0.18§
MAT	0.1	(0.1-0.1)	0.09†	0.23§
Total	13.2	(12.8-13.6)	0.12‡	0.24§

*Geometric mean (95% CI). †P<0.05. ‡P<0.01. §P<0.001.

differ between total, incident, or prevalent cases and controls (P for interaction all ≥0.41, results not shown).

In the univariate models, total lignan intake, age, weight, and use of antibiotics were significantly associated with plasma END (TABLE 5.3). In the full model only age and use of antibiotics remained significant, and the total explained variance was 2.1%. Total lignan intake, age, weight, current smoking, and frequency of defecation were associated with plasma ENL in both the univariate and the full model. The total explained variance of ENL concentrations was 12.7%.

We then further evaluated the dietary determinants of END and ENL after adjustment for age, weight, energy intake, use of antibiotics, current smoking, and frequency of defecation. Plasma ENL was associated with all four individual dietary lignans, whereas plasma END was only significantly associated with intake of LARI and PINO (TABLE 5.4). The increase in plasma END and ENL associated with 1-SD increase in intake of fiber or 1-SD increase in intake of vegetable protein was larger than that associated with 1-SD increase in total lignan intake. None of the other nutrients was significantly associated with plasma enterolignan concentrations. Wholegrain wheat bread was the only lignan-containing food for which the consumption was significantly associated with plasma END. Consumption of fruits, nuts and seeds, and wine were also significantly associated with plasma ENL concentrations. Beer consumption was significantly inversely associated with plasma ENL.

TABLE 5.3 Determinants of plasma enterolignans in 637 men and women in the POLIEP-study.

	Enterodiol					Enterolactone				
	Univariate model			Full model*		Univariate model			Full model*	
	β	*P*	Adj-R^2	β	*P*	β	*P*	Adj-R^2	β	*P*
Total plant lignans†	0.112	0.03	0.6	0.094	0.95	0.201	<0.001	2.2	0.179	<0.001
Age (y)	0.011	0.008	1.0	0.012	0.007	0.013	0.002	1.3	0.011	0.01
Sex (female vs. male)	0.080	0.44	0.1	0.012	0.93	0.140	0.18	0.1	0.158	0.19
Weight (kg)	-0.007	0.03	0.6	-0.006	0.11	-0.015	<0.001	2.7	-0.013	<0.001
Smoking (ex vs. never)	0.063	0.59	0.3	0.014	0.91	0.074	0.53	2.2	0.029	0.81
Smoking (current vs. never)	-0.200	0.12	0.3	-0.144	0.30	-0.438	<0.001		-0.348	0.009
Physical activity (score)	-0.007	0.89	0.2	-0.031	0.54	0.053	0.29	0.0	0.009	0.86
Antibiotics (ever vs. never)‡	0.237	0.05	0.5	0.275	0.03	-0.170	0.17	0.1	-0.119	0.33
Polyps (ever vs. never)	-0.092	0.36	0.0	-0.177	0.14	-0.033	0.75	0.1	-0.030	0.79
Indication for endoscopy										
complaints vs. other/unknown	-0.061	0.76	0.3	-0.124	0.55	0.204	0.32	0.2	0.208	0.30
screening vs. other/unkown	-0.011	0.96		-0.011	0.96	0.364	0.10		0.345	0.10
Frequency of defecation (n/wk)										
5-7 vs. > 7	0.108	0.33	0.2	0.118	0.31	0.533	<0.001	4.9	0.540	<0.001
1-5 vs. > 7	0.097	0.56		0.099	0.58	0.626	<0.001		0.656	<0.001
< 1 vs. > 7	0.315	0.39		0.303	0.41	1.293	0.001		1.389	<0.001
Diet change (yes vs. no)§	0.122	0.28	0.0	0.018	0.88	0.216	0.07	0.4	0.187	0.10

*Adjusted for all other variables in the table; Adjusted-R^2 = 2.1 for END and 12.7 for ENL. †Per 1-SD (345 µg/d). ‡Persons who used antibiotics in the previous calendar year were excluded. §Diet change because of bowel complaints.

TABLE 5.4 Dietary determinants of plasma enterolignans in 637 men and women in the POLIEP-study.

		END		ENL	
	mean ± SD	β*	*P*	β*	*P*
DIETARY LIGNANS (μg/d)					
LARI	488 ± 157	0.133	0.02	0.230	<0.001
PINO	362 ± 153	0.118	0.03	0.166	0.002
SECO	191 ± 64	0.052	0.35	0.176	0.001
MAT	9 ± 7	0.082	0.14	0.228	<0.001
SECO + MAT	200 ± 68	0.057	0.30	0.185	<0.001
Total	1045 ± 345	0.125	0.03	0.218	<0.001
ENERGY (kJ/d)†	8354 ± 2437	-0.076	0.16	-0.014	0.79
NUTRIENTS (g/d)					
Fiber	23 ± 7	0.149	0.03	0.296	<0.001
Carbohydrates	223 ± 69	-0.045	0.68	0.085	0.43
Vegetable protein	27 ± 9	0.178	0.03	0.357	<0.001
Animal protein	49 ± 17	-0.033	0.65	-0.120	0.10
Monounsaturated fat	30 ± 12	0.033	0.77	-0.028	0.80
Polyunsaturated fat	15 ± 6	0.121	0.11	0.106	0.15
Saturated fat	33 ± 12	-0.014	0.89	-0.152	0.14
Alcohol	13 ± 17	-0.019	0.74	-0.043	0.43
FOODS (g/d)					
Vegetables	114 ± 49	-0.005	0.92	0.043	0.40
Fruits	190 ± 57	0.015	0.78	0.131	0.01
Wholegrain wheat bread	56 ± 60	0.140	0.007	0.167	0.001
Other bread	77 ± 70	-0.001	0.11	-0.002	0.05
Nuts and Seeds	8 ± 16	0.071	0.18	0.163	0.002
Black tea	219 ± 224	0.077	0.14	0.077	0.13
Coffee	392 ± 293	-0.025	0.64	0.001	0.99
Wine	45 ± 80	0.016	0.75	0.126	0.01
Beer	88 ± 248	-0.003	0.96	-0.115	0.03

*Per 1-SD; Adjusted for age, weight, energy intake, use of antibiotics, current smoking, and frequency of defecation (4 categories: <1, 1-5, 5-7 or >7 times/week). †Adjusted for the same variables as above, except energy intake.

Discussion

In this study we found a modest positive association between plasma enterolignans and dietary lignan intake. The Spearman correlation coefficient of plasma END was 0.09 ($P = 0.03$), and was 0.18 for ENL ($P < 0.001$). As expected, the correlations improved (END, $r_s = 0.12$, $P = 0.002$; ENL, $r_s = 0.24$, $P < 0.001$) when lignan dose was used instead of lignan intake.

An important strength of our study was that we used a newly developed database[2] that included four major plant lignans, LARI, PINO, SECO, and MAT, whereas other studies included only SECO plus MAT. Furthermore, the lignan

database was especially developed to study the Dutch population. It included all major plant foods in the Netherlands habitually processed and prepared. In addition, all values in this database were obtained using one analytical method (LC-MS/MS) with an identical sample preparation for all food items.[13] Lignan intake in the year preceding endoscopy was estimated based on the habitual consumption of 178 food items in the Dutch EPIC-FFQ, which had a satisfactory reproducibility and relative validity compared to 12 monthly 24-h recalls for the main food groups contributing to lignan intake.[19] In addition, we have included both END and ENL, to assess the internal exposure (plasma concentrations), whereas most of the previous studies only included ENL.

A drawback of our study may be that the population studied included only persons who underwent endoscopy. Both cases and controls often had bowel complaints and/or a positive (family) history of abdominal polyps or cancer. Thus, results for this population may have differed from those for a healthy population. A total of 28% of the study participants indicated that they changed their diet because of their bowel complaints, but their plasma enterolignan levels were not significantly different from those of participants who did not change their diet. In addition, plasma enterolignan concentrations did not differ between persons who underwent endoscopy indicated by complaints, screening, or other/unknown reasons. And finally, the correlation between total lignan intake and plasma enterolignans did not differ between cases and controls. So, to the extent that we could verify this in our study, we found no evidence that the relation between plasma enterolignan levels and dietary lignan in our population differs from that in a healthy population and we have combined cases and controls in the analyses.

Vegetables, black tea, wholegrain bread, fruits, and wine were the most important lignan sources. Although vegetables and black tea contributed both more than 20% to the total lignan intake, they were not associated with plasma enterolignan concentrations. Consumption of wholegrain bread, fruits, nuts and seeds, and wine contributed less to the total lignan intake, but all were significantly associated with plasma enterolignan concentrations. This indicates that there are differences in the bioavailability from different foods or in the reliability of the intake measurements. Indeed, the relative validity of the EPIC-FFQ compared to 12 monthly 24 h recalls was relatively low for vegetables and higher for bread, fruits, and nuts and seeds.[19]

Although the correlation of lignan intake with the intake of dietary fiber is relatively strong ($r_s = 0.6$, $P < 0.001$; results not shown) and the variation in intake is similar, the increase in plasma enterolignans associated with 1-SD intake in fiber was larger than that associated with one 1-SD increase in total

lignan intake. Perhaps fiber-rich foods also contain other enterolignan precursors such as syringaresinol,[3] sesamin,[22] and lignin[23] that we did not take into account but that may also be converted to enterolignans. This is supported by the fact that the association between intake of dietary fiber and ENL was only slightly attenuated from $\beta = 0.296$ to $\beta = 0.215$ nM per 1-SD fiber intake ($P = 0.007$; results not shown), *i.e.*, it remained clearly positive when lignan intake was also included in the multivariate model.

Plasma END concentrations were positively associated with total lignan intake, age, and antibiotic use. We excluded participants who used antibiotics in the calendar year preceding the endoscopy. Therefore, it was remarkable that we found a significant positive association between plasma END and antibiotic use. Apparently, antibiotic use can have a long-term effect on plasma enterolignans and it does not affect plasma END and ENL concentrations in a similar way. A possible explanation for this finding is that the bacteria involved in the production of END and ENL from dietary precursors are not the same.[24,25] So far, one bacterial strain capable of catalyzing the oxidation of END to ENL has been identified.[26] Destruction of this strain by antibiotics may increase the relative concentration of END due to reduced conversion to ENL.

In agreement with previous studies, we found that plasma ENL concentrations were associated with age, and inversely associated with weight, frequency of defecation, and current smoking. Kilkinnen *et al.* found that serum ENL concentrations were higher in men and women with constipation.[27] They also found that serum ENL concentrations were associated with age and inversely associated with smoking and obesity in women, but not in men. Horner *et al.* reported that plasma ENL concentrations were associated with age and inversely associated with body mass index (BMI).[28]

The correlation between total dietary lignan intake and plasma ENL ($r_s = 0.18$, $P < 0.001$) was similar to correlations found previously for lignan density (SECO plus MAT/energy intake) and serum ENL concentrations ($r_s = 0.18$-0.19)[8,12] and for the correlation between intake of SECO plus MAT and urinary END plus ENL ($r_s = 0.17$-0.25).[11] However, it was stronger than the correlation between SECO plus MAT estimated using 12 monthly 24 h-recalls and plasma ENL reported by Bhakta *et al.* for South Asian ($r_s = 0.10$, $P = 0.1$) and native British ($r_s = 0.08$, $P = 0.6$) women in the UK.[10]

In our study, associations between lignan intake and plasma enterolignans were stronger when we included four dietary lignans than when we included only SECO plus MAT. This indicates that inclusion of four dietary lignans better reflects the exposure to enterolignans than only SECO plus MAT. In the study of Hedelin *et al.,* seven additional dietary lignan were included,

but the correlation between lignan intake and serum ENL was not stronger than when only SECO and MAT were included.[12] However, data on these additional lignans were available only for milk, bread, and cereal products. It should also be noted that with the inclusion of four dietary lignans and two lignan metabolites, both the dietary lignan intake and plasma enterolignan concentrations may still be underestimated, because more enterolignan precursors,[3,22,23] as well as lignan metabolites,[29] have been identified. In addition, the estimation the dietary lignan exposure could be improved by taking into account seasonal and varietal variation in lignan content and differences in bioavailability of lignans from different foods.[30] Food processing may affect both the lignan content and bioavailability from foods, *e.g.* crushing or milling substantially improved the bioavailability of lignans from flaxseed.[31]

We assumed that the plasma concentrations of END and ENL reflect steady-state concentrations because enterolignans are eliminated slowly, their precursors are present in many foods and beverages, and they are eaten several times a day.[32] Thus, one plasma sample will probably reflect exposure for a longer period. Indeed, the reliability coefficient estimated from three yearly serum samples was moderately high for ENL (0.55; 95% CI: 0.41-0.69), but lower for END (0.37; 95% CI: 0.21-0.53).[33]

Several researchers have pointed out the large intra- and inter-individual variation in plasma enterolignan concentrations due to differences in the composition of the colonic microflora.[34,35] Knowledge on the bacterial strains responsible for the conversion of dietary lignans to enterolignans is emerging,[24,25] but so far it is not possible to take into account the microflora composition as a factor modulating the relation between plasma and dietary lignans. We could adjust for habitual diet composition, sex, age, previous use of antibiotics, smoking, presence of polyps, and frequency of defecation, which all may affect the composition of the microflora. However, when we included these nondietary factors, in the multivariate adjusted models, the explained variance in plasma END and ENL concentrations remained low (2% and 13% respectively).

In summary, we found a modest positive association between dietary lignan intake and plasma enterodiol and enterolactone. When four dietary lignans were included, the associations between dietary lignan intake and plasma enterolignan concentrations were stronger than when only SECO plus MAT were included. However, even when we also included age, weight, energy intake, previous use of antibiotics, current smoking, and frequency of defecation in the regression models the explained variance in plasma END and ENL remained low.

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Relation between lignan intake and disease-specific and all-cause mortality

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Abstract:
Plant lignans are converted to enterolignans that have antioxidant and weak estrogen-like activities, and may therefore they may lower cardiovascular and cancer risk. We investigated whether the intake of four plant lignans lariciresinol (LARI), pinoresinol (PINO), secoisolariciresinol (SECO), and matairesinol (MAT) were inversely associated with coronary heart disease (CHD), cardiovascular diseases (CVD), cancer, and all-cause mortality. The Zutphen Elderly Study is a prospective cohort study in which 570 men aged 64-84 y were followed for 15 y. We recently developed a database, and used it to estimate the dietary intakes of four plant lignans. Lignan intake was related to mortality with the use of Cox proportional hazards analysis.
The median total lignan intake in 1985 was 977 μg/d. Tea, vegetables, bread, coffee, fruit, and wine were the major sources of lignans. The total lignan intake was not related to mortality. However, the intake of MAT was inversely associated with CHD, CVD, and all-cause mortality ($P \leq 0.05$ for all), and cancer ($P = 0.06$). Multivariate-adjusted RRs (95% CI) per 1-SD increase in intake were 0.72 (0.53-0.98) for CHD, 0.83 (0.69-1.00) for CVD, 0.81 (0.65-1.00) for cancer, and 0.86 (0.76-0.97) for all-cause mortality. In conclusion, total lignan intake was not associated with mortality. The intake of MAT was inversely associated with mortality due to CHD, CVD, cancer, and all causes. We can not rule out that the inverse association between matairesinol intake and mortality is due to an associated factor, such as wine consumption.

Keywords:
lignans, diet, phytoestrogens, secoisolariciresinol, matairesinol, lariciresinol, pinoresinol, cancer, cardiovascular diseases, mortality, cohort study, elderly, Netherlands

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Introduction

Lignans are biphenolic compounds in plant foods that belong to the phytoestrogens group. Tea, coffee, wholegrain products, vegetables, fruit, nuts and seeds are dietary sources of lignans.[1,2] Flaxseed has an exceptionally high lignan concentration. Plant lignans can be converted by intestinal bacteria into the so-called enterolignans: enterolactone and enterodiol. It has been shown that besides the known enterolignan precursors secoisolariciresinol (SECO) and matairesinol (MAT), lariciresinol (LARI) and pinoresinol (PINO) were also efficiently converted.[3]

There are several mechanisms by which phytoestrogens may protect against cardiovascular diseases (CVD) and cancer. Enterolignans have weak estrogen-like activity. They may bind to the estrogen receptors α and β,[4] which are expressed in various tissues. They also exert effects via non-estrogen receptor mediated mechanisms,[5] such as the inhibition of several enzymes (*e.g.* aromatase and 5α-reductase) and the stimulation of the production of sex hormone-binding globulin (SHBG). In addition, plant lignans, and to a lesser extent, enterolignans have antioxidant activity.[6]

The epidemiological evidence of the potential protective effects of lignan intake is limited. Previous studies only included SECO and MAT in relation to CVD, and these studies yielded conflicting results.[7-10] The relation between lignan intake and cancer has mainly been studied with respect to hormone-dependent cancers. In women, protection against ovarian, endometrial, and thyroid cancer was observed in case-control studies.[11] Conflicting results were obtained for the relation between intake of SECO plus MAT and breast cancer.[11-13] In men, the intake of SECO plus MAT was not associated with testicular cancer[14] or prostate cancer[15] in case-control studies. Intake of SECO plus MAT was significantly inversely associated with lung cancer in men but not in women.[16]

Previously, data on only SECO and MAT in foods were available. We have developed an LC-MS method to measure LARI and PINO in addition to SECO and MAT in foods and beverages;[17] and we constructed a lignan database of plant foods commonly consumed in the Netherlands.[2] With this database, we evaluated whether a higher intake of plant lignans is associated with a lower risk of CHD, CVD, cancer, and all-cause mortality in a cohort of Dutch elderly men.

Subjects and Methods

Study population

The Zutphen Elderly Study is a prospective cohort study on risk factors for chronic diseases in elderly men. It is an extension of the Zutphen Study, the

Dutch contribution to the Seven Countries study.[18] In 1985, men from the original Zutphen cohort who were still alive (n = 554) were invited to participate in the Zutphen Elderly Study. In addition, a random sample of men of the same age living in Zutphen but who had not been included in the original cohort was selected. In total, 1266 men aged 65-84 y were invited, of which 939 (74%) agreed to participate.[19] Complete information on baseline diet, prevalence of chronic disease, and other important risk factors was available for 787 men. We excluded men with a history of myocardial infarction (n = 97), stroke (n = 25), heart failure (n = 28), diabetes (n = 41) or cancer (n = 56). One man consumed flaxseed as a supplement, and was excluded because of his extremely high lignan intake (≈28 mg/d in 1985). After these exclusions 570 men remained in the analyses. The study was approved by the Medical Ethics Committee of the University of Leiden, The Netherlands. Informed consent was obtained from all participants.

Data collection

Dietary surveys and medical examinations were conducted between March and June in 1985, 1990 and 1995. Information on the habitual diet of the participants was collected by experienced dietitians who used a cross-check dietary history method adapted to usual Dutch diets.[20] Participants were interviewed, in the presence of the person who usually prepared the meals, about their usual food consumption on weekdays and weekends. The estimated consumption of foods during 1 d or 1 wk was compared with the estimated quantities of food bought for the whole family. Discrepancies were discussed with the participant, and resolved accordingly. The food consumption data were encoded by the dietitians and converted into energy and nutrients using the 1985 release of the Dutch Food Composition Table updated with 2001 data for β-carotene, folate, vitamin E and dietary fiber. The intakes of *trans* fatty acids, α-linolenic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were estimated previously by Oomen *et al.*[21] the intakes of flavonols and flavones were estimated by Hertog *et al.*[19] and the intakes of catechins were estimated by Arts *et al.*[22] Lignan intake was estimated using a recently developed database including contents of LARI, PINO, SECO, and MAT of commonly consumed Dutch plant foods.[2] Lignans were measured after hydrolysis of lignan glycosides, and thus lignan contents included both aglycones and glycosides. In this thesis, the lignan contents are expressed as the aglycone weight of the molecule.

Trained physicians measured height and weight, and blood pressure measurements were taken while the subjects were lying in supine position.

Nonfasting serum total and HDL cholesterol were determined enzymatically.[23] Physical activity was estimated using a validated questionnaire, originally designed for retired men.[24] It included questions on walking, bicycling, hobbies, gardening, odd jobs, and sports. Information on smoking was obtained by using a standardized questionnaire and on alcohol consumption by the cross-check dietary history method.

Case ascertainment

Municipal registries were checked at 5-y intervals from 1985 to July 2006 to ascertain whether participants had died in the interim. Causes of death were obtained from various sources, mainly hospital discharge data, and supplemented information was obtained from the Netherlands Cancer Registry, general practitioners, and until 1990, from Statistics Netherlands, Voorburg. All information was used to establish the contributory causes of death. Because this is often difficult to ascertain in elderly people, the primary, as well as secondary and tertiary causes of death were used in the statistical analyses. The final coding of the causes of death was performed by one clinical epidemiologist, according to the 9th revision of the International Classification of Causes of Death (ICD-9). We classified ICD codes, 410-414 as CHD, 390-459 as CVD and codes 140-208 as cancer. One man was lost to follow-up in 1991, and his follow-up was censored at 30 June 1991. Information on the causes of death was missing for two men, and their follow-up was censored at their date of death.

Statistical analysis

The subjects were divided into tertiles of total lignan intake at baseline. To test for differences in baseline characteristics we used analysis of variance for normally distributed variables, Kruskal-Wallis test for skewed distributed variables and the chi-square test for categorical variables. Spearman's rank order correlations between the intake of lignans and the intakes of other nutrients and lignan-containing foods were calculated. Rate ratios (RRs) and 95% confidence intervals (CIs) per 1-SD increase in intakes of total and individual lignans were estimated by hazard ratios obtained from Cox proportional hazards models. For comparison with the literature, we also determined the RRs per 1-SD increase in intakes of SECO plus MAT.

To represent long-term exposures, we ascertained the cumulative averages of lignan intake, other dietary variables, and physical activity.[25] Thus, the mortality from 1985 to 1990 was related to the estimated values in 1985, and that between 1990 and 1995 and between 1995 and 2000 was related to the

average of the values in 1985 and 1990 and the average of the values in 1990 and 1995, respectively. The follow-up rates for the dietary questionnaires in 1990 and 1995 were 82 and 65%, respectively, of the men who were still alive at those dates. When updated dietary data were not available, the last available value was used. For smoking the most recent data were used. Smoking status was included in the models as three categories (current, former, never), duration and amount were calculated from the questionnaire, and entered as a continuous variable.

Besides age-adjusted analyses we used two multivariate models. In model 1, we adjusted for age, smoking status, amount and duration of smoking, physical activity, and energy intake. In model 2 (full model), we also adjusted for intakes of dietary fiber and β-carotene.

Initially, BMI, alcohol consumption (> 30 g/d, ≤ 30 g/d or no alcohol), socioeconomic status (4 categories, based on occupation), and intakes of saturated fatty acids, *trans* fatty acids, α-linolenic acid, EPA, DHA, dietary cholesterol, vitamins C and E, and folate were included as potential confounders, but they were removed because they had *P* values >0.3 for each endpoint. Re-entering the previously removed variables one by one in model 2 did not change any of the RRs (< 5% change).

Smokers have greater oxidative stress than do nonsmokers, and thus the antioxidant effects of lignans might be more marked in the former than in the latter. We therefore performed analyses stratified for smoking status (current, former, or never). The intake of dietary fiber may influence the bioavailability of lignans, perhaps because of effects on intestinal transit time. Therefore, we also stratified for fiber intake above or below the median (23 g/d). All statistical analyses were conducted using SAS statistical analysis package (version 9.1; SAS Institute, Inc, Cary, NC).

Results

The median intake of total lignans in 1985 was 977 μg/d (mean 1037 μg/d) (TABLE 6.1). Of the individual lignans, LARI contributed most (48%) to the mean lignan intake, and PINO (36%), SECO (15%), and MAT (1%) followed. The intakes of the individual lignans were moderately to highly correlated (r_s = 0.4-0.8; $P < 0.001$). The major sources of lignans for this population were tea (28%), vegetables (27%), bread (14%), coffee (10%), fruit (9%), and wine (1%). The median lignan intake was 1000 μg/d in 1990 and 1053 μg/d in 1995. Spearman's correlation coefficient between the total lignan intake in 1985 and 1990 was 0.51 and that between 1985 and 1995 was 0.47 ($P < 0.001$ for both).

TABLE 6.1 Contribution of different food groups to lignan intake in 1985 in 570 men in the Zutphen Elderly Study.*

	% Contribution†				
	LARI	PINO	SECO	MAT	Total
Tea	25	37	14	47	28
Vegetables	29	28	21	3	27
Bread	14	14	10	19	14
Coffee	9	1	32	9	10
Fruit	10	9	4	10	9
Wine	0	0	4	7	1
Other	12	9	15	5	11

*Mean ± SD and (median) lignan intakes were 494 ± 169 (476) μg/d for LARI, 374 ± 210 (334) μg/d for PINO, 158 ± 51 (152) μg/d for SECO, 12 ± 7 (11) μg/d for MAT, and 1037 ± 397 (977) μg/d total. Lignans were measured after hydrolysis of lignan glycosides. Thus, lignan contents include both aglycones and glycosides. Lignan contents are expressed as the aglycone weight of the molecule. †Percentage contribution to the mean population intake of the various lignans.

Men in the highest tertile of total lignan intake were less likely to smoke, were more physically active, and had a higher energy intake than men in the lowest tertile (TABLE 6.2). After adjustment for energy intake, total lignan intake was positively correlated with intakes of fiber (r_s = 0.51), vitamin C (r_s = 0.39), folate (r_s = 0.37), and β-carotene (r_s = 0.32), and was weakly and inversely associated with the intakes of saturated fatty acids (r_s = –0.13), *trans* fatty acids (r_s = –0.09), and dietary cholesterol (r_s = –0.10). Tea was the major source of lignans. As a consequence, lignan intake was correlated with intakes of flavonols and flavones (r_s = 0.70), and catechins (r_s = 0.62), because in this population tea also is the major source of these bioactive compounds.

After 15 y of follow-up (5888 person-years), 392 of the 570 men had died. Of these 392 men, 84, 197 and 125 had CHD, CVD, and cancer, respectively, as primary, secondary or tertiary cause of death. After multivariate adjustment total lignan intake was not associated with cause-specific or all-cause mortality (TABLE 6.3). To compare our results with previous data, we have calculated the RRs per 1-SD (55 μg/d) increase in intake of SECO plus MAT. The RRs (95% CI) after full adjustment were 0.81 (0.58-1.12) for CHD, 0.86 (0.69-1.06) for CVD, 0.87 (0.68-1.11) for cancer, and 0.95 (0.83-1.09) for all-cause mortality. We did not find significant lignan intake × smoking interaction or lignan intake × fiber interaction for any of the endpoints (*P* for interaction all ≥ 0.44 for all, results not shown).

After full adjustment (model 2), MAT was the only individual lignan whose intake was significantly associated with mortality. The intake of MAT was significantly inversely associated with CHD, CVD, and all-cause mortality,

TABLE 6.2 Characteristics of 570 men in the Zutphen Elderly Study by tertile of total lignan intake in 1985.*

	T1 (low) (n = 190)	T2 (n = 190)	T3 (high) (n = 190)	*P*†
Total lignans (mg/d)‡	693 (598-771)‡	977 (898-1043)	1379 (1212-1599)	
LARI (mg/d)‡	343 (302-383)	476 (436-518)	640 (572-751)	
PINO (mg/d)‡	201 (160-243)	340 (291-386)	538 (441-664)	
SECO (mg/d)‡	123 (106-145)	150 (128-169)	184 (161-217)	
MAT (mg/d)‡	7 (4-9)	11 (9-13)	16 (161-217)	
Age (years)	71 ± 5§	71 ± 5	71 ± 5	0.49
BMI (kg/m^2)	25.2 ± 3.2	25.6 ± 3.1	25.9 ± 3.0	0.11
Current smokers (%)	43	35	21	<0.001
Cigarette amount (n cigarettes/d)$	15 (8-20)	10 (6-20)	10 (6-20)	0.04
Duration of smoking (y)‡§	48 (36-54)	49 (39-55)	40 (28-50)	<0.001
Alcohol users (%)	74	76	78	0.63
SES (% high)	29	24	35	0.10
Physical activity (h/week)‡	8.8 (4.0-16.4)	10.0 (5.0-18.9)	11.7 (5.9-21.0)	0.01
Systolic blood pressure (mm Hg)	151 ± 20	150 ± 21	152 ± 22	0.47
Diastolic blood pressure (mm Hg)	85 ± 11	86 ± 11	86 ± 12	0.77
Total cholesterol (mmol/L)	6.08 ± 1.05	6.05 ± 1.10	6.17 ± 1.00	0.52
HDL cholesterol (mmol/L)	1.15 ± 0.33	1.11 ± 0.28	1.15 ± 0.26	0.26
Nutrient intakes#				
Energy intake (MJ/day)	8.9 ± 2.0	9.8 ± 2.2	10.1 ± 2.1	<0.001
Saturated fat (g/d)	47 ± 8	44 ± 9	44 ± 9	0.01
Monounsaturated fat (g/d)	42 ± 9	39 ± 9	39 ± 10	0.01
Trans fat (g/d)‡	12 ± 5	11 ± 6	11 ± 6	0.19
Polyunsaturated fat (g/d)	17 ± 7	17 ± 7	15 ± 6	0.08
EPA and DHA (g/d)‡	0.11 (0.03-0.20)	0.09 (0.03-0.20)	0.09 (0.02-0.19)	0.50
α-Linolenic acid (g/d)	1.34 ± 0.34	1.30 ± 0.31	1.32 ± 0.38	0.56
Dietary cholesterol (mg/d)	354 ± 89	340 ± 100	334 ± 113	0.15
Fiber (g/day)	22 ± 5	24 ± 5	28 ± 6	<0.001
Folate (mg/day)	170 ± 51	180 ± 45	208 ± 58	<0.001
Vitamin C (mg/d)	75 ± 37	94 ± 41	109 ± 45	<0.001
Vitamin E (mg/d)‡	6.7 (4.9-19.6)	8.6 (5.6-23.9)	8.8 (6.1-14.8)	0.52
β-carotene (mg/d)	2.1 ± 1.2	2.4 ± 1.2	3.2 ± 1.9	<0.001
Flavonols and flavones (mg/d)	16 ± 8	24 ± 9	35 ± 13	<0.001
Catechins (mg/d)	40 ± 26	67 ± 31	101 ± 53	<0.001

*Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. †ANOVA for normally distributed variables, Kruskal-Wallis for skewed variables and chi-square test for categorical variables. ‡Median; 25th-75th percentile in parentheses (all such values). §Mean ± SD (all such values). $Excluding never smokers. #Adjusted for energy intake by using the residual method.

TABLE 6.3 Rate ratios (RR) for mortality from coronary heart disease (CHD), cardiovascular disease (CVD), cancer, and all causes per 1-SD increase in intake of lignans in 570 men in the Zutphen Elderly Study.

	Total lignan intake	*P*	LARI	*P*	PINO	*P*	SECO	*P*	MAT	*P*
CHD (n = 84)*										
Age-adjusted	0.77 (0.59-1.01)†	0.06	0.75 (0.58-0.98)	0.03	0.84 (0.64-1.10)	0.20	0.79 (0.61-1.02)	0.07	0.68 (0.52-0.90)	0.006
Model 1‡	0.77 (0.57-1.03)	0.07	0.75 (0.57-0.99)	0.04	0.85 (0.63-1.13)	0.25	0.78 (0.59-1.03)	0.08	0.68 (0.51-0.91)	0.009
Model 2§	0.85 (0.60-1.20)	0.35	0.82 (0.57-1.17)	0.27	0.93 (0.68-1.26)	0.62	0.84 (0.61-1.17)	0.31	0.72 (0.53-0.98)	0.03
CVD (n = 197)										
Age-adjusted	0.82 (0.69-0.97)	0.02	0.82 (0.69-0.96)	0.02	0.87 (0.73-1.04)	0.12	0.80 (0.68-0.95)	0.01	0.76 (0.64-0.90)	0.001
Model 1‡	0.86 (0.72-1.04)	0.12	0.86 (0.72-1.02)	0.09	0.92 (0.77-1.11)	0.40	0.81 (0.68-0.97)	0.02	0.81 (0.68-0.96)	0.02
Model 2§	0.97 (0.77-1.22)	0.79	0.96 (0.76-1.22)	0.73	1.02 (0.83-1.25)	0.86	0.88 (0.71-1.08)	0.23	0.83 (0.69-1.00)	0.05
Cancer (n = 125)										
Age-adjusted	0.86 (0.70-1.06)	0.16	0.86 (0.70-1.06)	0.15	0.87 (0.70-1.09)	0.22	0.92 (0.76-1.12)	0.42	0.76 (0.62-0.94)	0.01
Model 1‡	0.93 (0.75-1.16)	0.52	0.93 (0.75-1.15)	0.52	0.95 (0.76-1.19)	0.66	0.93 (0.77-1.13)	0.48	0.83 (0.68-1.02)	0.08
Model 2§	0.89 (0.68-1.16)	0.40	0.88 (0.67-1.16)	0.37	0.94 (0.74-1.19)	0.61	0.89 (0.70-1.14)	0.36	0.81 (0.65-1.00)	0.06
All cause (n = 392)										
Age-adjusted	0.82 (0.72-0.92)	0.001	0.81 (0.72-0.91)	<0.001	0.84 (0.74-0.95)	0.007	0.89 (0.80-1.00)	0.05	0.78 (0.69-0.87)	<0.001
Model 1‡	0.89 (0.78-1.01)	0.06	0.88 (0.77-0.99)	0.04	0.92 (0.80-1.04)	0.18	0.92 (0.82-1.03)	0.15	0.85 (0.75-0.95)	0.005
Model 2§	0.93 (0.80-1.09)	0.36	0.91 (0.78-1.07)	0.27	0.96 (0.83-1.10)	0.54	0.97 (0.85-1.11)	0.69	0.86 (0.76-0.97)	0.02

*Number of deaths (for all such). †95% CI in parentheses (all such values). ‡Adjusted for age, smoking status (current/former/never), amount and duration of smoking, physical activity, and energy intake. §Adjusted for same covariates as model 1 plus intake of β-carotene and dietary fiber.

TABLE 6.4 Rate ratios (RR) for mortality from coronary heart disease (CHD), cardiovascular diseases (CVD), cancer, and all causes per 1-SD increase in intake of MAT intake with additional adjustments for factors associated with MAT intake in 570 men in the Zutphen Elderly Study.

	CHD	*P*	CVD	*P*	Cancer	*P*	All cause	*P*
Model 2*	0.72 (0.53-0.98)†	0.03	0.83 (0.69-1.00)	0.05	0.81 (0.65-1.00)	0.06	0.86 (0.76-0.97)	0.02
Model 2 + vegetables	0.72 (0.54-0.98)	0.04	0.83 (0.69-1.00)	0.05	0.81 (0.65-1.01)	0.06	0.86 (0.76-0.97)	0.02
Model 2 + bread	0.70 (0.52-0.96)	0.03	0.82 (0.68-0.99)	0.04	0.81 (0.65-1.01)	0.06	0.84 (0.74-0.96)	0.01
Model 2 + coffee	0.72 (0.53-0.98)	0.04	0.83 (0.69-1.00)	0.05	0.81 (0.64-1.01)	0.06	0.87 (0.77-0.99)	0.03
Model 2 + fruit	0.73 (0.54-0.99)	0.04	0.84 (0.70-1.01)	0.06	0.83 (0.67-1.02)	0.08	0.86 (0.76-0.98)	0.02
Model 2 + wine	0.85 (0.61-1.18)	0.32	0.88 (0.71-1.08)	0.22	0.86 (0.67-1.10)	0.24	0.88 (0.77-1.02)	0.09
Model 2 + alcohol‡	0.73 (0.54-0.99)	0.04	0.83 (0.69-0.99)	0.04	0.82 (0.66-1.02)	0.08	0.86 (0.76-0.97)	0.02
Model 2 + SES§	0.77 (0.56-1.06)	0.11	0.83 (0.68-1.00)	0.05	0.81 (0.65-1.02)	0.07	0.88 (0.77-1.00)	0.05

*Adjusted for age, smoking status (current/former/never), amount and duration of smoking, physical activity, energy intake, β-carotene, and dietary fiber. †RR, 95% CI in parentheses (all such values). ‡Alcohol consumption 0, ≤ 30, or > 30 g/d. §Socioeconomic status, 4 categories based on (former) profession.

and borderline (P = 0.06) with cancer mortality (TABLE 6.3). When we did not include the adjustment for β-carotene and dietary fiber (model 1) we found significant associations between LARI intake and both CHD and all-cause mortality, and between SECO intake and CVD mortality.

Tea is the major contributor (47%) to the intake of MAT. Since tea is inversely associated with CHD risk in this population,[19,22] we evaluated whether the protective effect observed for MAT could be attributed to other components in tea, *e.g.* catechins or flavonols. Because of multi-collinearity, it was not possible to adjust for these factors in the regression analyses. Therefore, after adjustment for tea consumption, we investigated whether MAT from sources other than tea was associated with mortality. For MAT from sources other than tea, the RRs per 1-SD increase in intake did not differ significantly from those for total MAT intake. In the fully adjusted models RRs (95% CI) were 0.71 (0.52-0.97) for CHD, 0.79 (0.65-0.95) for CVD, 0.74 (0.59-0.94) for cancer, and 0.85 (0.75-0.96) for all-cause mortality.

We also evaluated the effect of adjustment for the other major dietary sources of MAT (*i.e.* vegetables, bread, coffee, fruit and wine) by adding these variables one by one to the fully adjusted models. None of these variables substantially changed the RRs (<5% change in RRs), except wine consumption (TABLE 6.4). After additional adjustment for wine consumption the RR (95% CI) for CHD was attenuated from 0.72 (0.53-0.98) to 0.85 (0.61-1.18). The RRs for the other endpoints were also attenuated. Because wine consumption was associated with alcohol consumption and socioeconomic status (SES), we evaluated the effect of adjusting for these variables. Additional adjustment for alcohol intake (0, ≤ 30, > 30 g/d) did not change the associations. Additional adjustment for SES, attenuated the association between intake of MAT and CHD mortality, but the attenuation was less than that observed after adjustment for wine consumption.

Discussion

In this prospective study of elderly men who were free of CVD and cancer at baseline, total lignan intake was not associated with mortality, after adjustment for potential confounders. However, the intake of MAT was significantly and inversely associated with CHD, CVD, and all-cause mortality and tended toward a significant association with cancer mortality. Additional adjustment for the consumption of wine, an important source of MAT, attenuated these associations, especially the association with CHD mortality.

In previous epidemiologic studies, only the intakes of SECO and MAT were investigated. In the current study, SECO and MAT contributed 16% to the mean

total lignan intake. Thus, the inclusion of four dietary lignans gives a better representation of the exposure to enterolignans, than does SECO and MAT alone. Although arctigenin, 7-hydroxy-MAT, syringaresinol,[3] sesamin,[26] and lignins[27] have also been identified as enterolignan precursors, they appear to be less important than the four selected precursors, because their degree of conversion is lower, they are not commonly present in foods, or both.

An alternative to the quantification of precursors in the diet is the measurement of enterolignan concentrations in plasma or urine. This approach has the advantage that it takes into account all precursors, as well as their bioavailability, but it also has the disadvantage is that those biomarkers reflect mostly short-term intake.[28] The dietary history method used in this study reflects habitual dietary intake, which is more relevant to mortality risk.

So far, only a few studies have investigated the relation between lignan intake and CVD. In elderly women in the Netherlands, lignan intake was inversely associated with incident CHD only in former and current smokers.[7] This result was not confirmed in the present study. The results with respect to lignan intake are in contrast with those of a Finnish study of plasma enterolactone concentrations and CVD. In that study, men in the highest quartile of plasma enterolactone had a significantly lower risk of incident myocardial infarction RR (95% CI): 0.35 (0.14-0.88),[29] and of CHD 0.44 (0.20-0.96) and total CVD 0.55 (0.29-1.01) mortality.[30] However, in this study some important potential confounders, such as physical activity, were not considered.

Especially for men, data on the relation between lignan intake and cancer are still limited. No association was found between lignan intake and testicular cancer,[14] or prostate cancer.[15] In a case-control study the intake of SECO plus MAT was inversely associated with lung cancer in men, but not in women. We did not observe an inverse association between total lignan intake and cancer mortality, although men with a high intake of MAT tended to have lower cancer mortality.

We noted consistent associations between the intake of MAT and the various endpoints. This is remarkable, because the intake of MAT is very low compared with that of the other lignans. We assumed that lignans have to be converted to enterolignans in order to be physiologically active. Under this assumption the prominent role of MAT can be explained only if MAT is more efficiently converted. However, an *in vitro* fermentation experiment[3] and a subsequent study in rats,[31] did not indicate a higher degree of conversion of MAT compared with the other three major precursors. So, our data suggest that enterolignans may not be the physiologically active compounds.

Support for a direct role of MAT comes from studies that showed that

plant lignans were absorbed as such.[32] In an *in vitro* study, MAT inhibited matrix metalloproteinase 7 (MMP-7), whereas SECO showed no inhibition.[33] MMP-7 is thought to play a role in tumor invasion and metastasis as well as in atherosclerosis.[34] Inhibition of MMP-7 could be a mechanism for a protective effect of MAT against both cancer and CVD, and it may also explain the results from a German case-control study in which breast cancer risk was inversely associated with MAT intake, but not with SECO or total lignan intake.[35] However, it should be noted that the concentrations used *in vitro* are much higher than the expected physiologic lignan concentrations.

Because of the observational nature of the present study, residual confounding can not be fully excluded as an explanation for the observed associations. We have adjusted for age, smoking, physical activity, and intake of energy, β-carotene, and dietary fiber. To gain more insight in potential sources of residual confounding, we have evaluated the effect of adjusting for the major dietary sources of MAT. The associations between intake of MAT and mortality were substantially attenuated only by adjustment for wine consumption. The RR for CHD was particularly attenuated. This change could not be attributed to alcohol because adjustment for alcohol intake did not change the RRs. Thus, bioactive compounds other than alcohol found in wine may explain the inverse associations found for MAT.

In summary, we observed no association between total lignan intake and CHD, CVD, cancer, and all-cause mortality in a population of elderly men. Only the intake of MAT, a minor lignan, was inversely associated with all endpoints considered, which suggests that enterolignans are not the active compounds. Associations with MAT were attenuated and no longer statistically significant after additional adjustment for wine consumption. Thus, either MAT or a factor associated with MAT intake, such as wine consumption, may be responsible for the inverse associations of MAT with CVD and cancer.

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General discussion

7

Background

The aim of the project described in this thesis was to develop a method for the quantification of four plant lignans in foods, to determine the contents of these lignans in Dutch plant foods, to estimate the lignan intake in the Netherlands, to identify the major dietary sources of lignans, and to estimate the associations of lignan intake with plasma enterolignans and with the risk of cardiovascular and cancer mortality.

Main findings

The main findings of the studies described in this thesis, are summarized in TABLE 7.1. In order to get a comprehensive picture of the intake of the most relevant enterolignan precursors in the Netherlands, we first had to develop a method for the quantification of the lignans lariciresinol (LARI), pinoresinol (PINO), secoisolariciresinol (SECO), and matairesinol (MAT) in a wide range of foods (CHAPTER 2). The lignan database that we subsequently constructed is the first with four major enterolignan precursors covering all commonly consumed plant foods in the Netherlands (CHAPTER 3). In a representative sample of the Dutch population (National Food Consumption Survey), we showed that the two new enterolignan precursors LARI and PINO that had previously not been included in databases, account for 75% of the lignan intake in the Netherlands. The median total lignan intake was approximately 1 mg/day (TABLE 7.2). The median intake of LARI was 465 µg/d, of PINO 306 µg/d, of SECO 167 µg/d and of MAT 9 µg/d. Remarkably, beverages such as coffee, tea and beer, were the most important lignan sources. This contrasts with the traditional view that fiber-rich products are the most important lignan sources. Other important sources were vegetables, nuts and seeds, bread, and fruits (CHAPTER 4).

In the POLIEP-study (CHAPTER 5), we found a modest correlation between total lignan intake and plasma enterolignans ($r_s = 0.18$, $P < 0.001$). The plasma enterolignan concentration was associated with intake of dietary fiber and vegetable protein, but not with intake of other macronutrients. The relation between lignan intake and plasma END was modulated by age, and previous use of antibiotics, whereas for ENL it was modulated by weight, current smoking and the frequency of defecation. However, even when these non-dietary factors were included in the regression models, the explained variance remained low, 2% for END and 13% for ENL, respectively.

In the Zutphen Elderly Study, total lignan intake was not associated with mortality (CHAPTER 6). However, interestingly, the intake of MAT was inversely associated with disease specific and all-cause mortality. The multivariate-

TABLE 7.1 Main findings of the studies described in this thesis.

Ch	Objectives	Study	Results/Conclusions
2	To develop an analytical method, to quantify four lignans in a wide range of foods	A LC-MS/MS method was developed and optimized using test products such as flaxseed. The method exists of alkaline methanolic extraction, followed by enzymatic hydrolysis using *H. pomatia*. After ether extraction, samples were analyzed, and quantified against SECO-d_8 and MAT-d_6. *H. pomatia* was selected from several enzymes, based on its ability to hydrolyze isolated lignan glucosides.	– The yield of the methanolic extraction increased with up to 81% when it was combined with alkaline hydrolysis. – Detection limits were 4-10 µg/(100g dry weight) for solid foods and 0.2-0.4 µg/100 mL for beverages. – Within-run and between-run coefficients of variation were 6-21 and 6-33%, respectively. – Recovery of lignans was satisfactory (73-123%), except for MAT added to bread (51-55%).
3	To develop a database with lignan contents of commonly consumed plant foods.	Lignans were quantified in composite samples of 83 solid foods and 26 beverages commonly consumed in the Netherlands.	– Almost all plant foods contained lignans. – Flaxseed (± 300 000 µg/100 g) was the most abundant lignan source; it contained mainly SECO. Other important lignan sources were sesame seeds, grain products, vegetables, fruits, and red wine. *Brassica* vegetables had unexpectedly high values of LARI and PINO.
4	To estimate the habitual intake of plant lignans in the Netherlands; and to identify the major dietary sources of lignans.	Lignan intake was estimated in a representative sample of 4660 men and women, aged 19-97 y, participating in the Dutch National Food Consumption Survey, carried out in 1997-1998.	– The median total lignan intake was 979 µg/d (range 43-77 584 µg/d) – LARI + PINO contributed 75% to lignan intake, whereas SECO + MAT only 25%. – The major food sources of lignans were beverages (37%), vegetables (24%), nuts and seeds (14%), bread (9%) and fruits (7%).

TABLE 7.1 Continued.

Ch	Objectives	Study	Results/Conclusions
5	To study the association between plasma and dietary lignans, and to identify dietary and non-dietary determinants of plasma lignans.	The relation between dietary and plasma lignans was studied cross-sectionally in an endoscopy-based population of 637 men and women, aged 19-75 y, who did not use antibiotics in the preceding calendar year	– The correlation between total dietary lignan intake and plasma enterolignans was modest (r_s = 0.18). – Even when dietary and non-dietary determinants were included in the regression models the explained variance remained low (2% for END and 13% for ENL).
6	To study the association between lignan intake of 4 plant lignans and coronary heart disease (CHD), cardiovascular diseases (CVD), cancer, and all-cause mortality	The relation between lignan intake and mortality was studied in the Zutphen Elderly Study, a prospective cohort study in which 570 men aged 64-84 y were followed for 15 y, using Cox proportional hazards analysis.	– The total lignan intake was not related with mortality. However, the intake of MAT was inversely associated with CHD, CVD, cancer, and all-cause mortality. Adjusted rate ratios (95% CI) varied between 0.72 (0.53-0.98) for CHD and 0.86 (0.76-0.97) for all-cause mortality.

TABLE 7.2 Summary of the estimates of lignan intake in the studies described in this thesis.*

Study	Population†	LARI	PINO	SECO	MAT	Total
National Food	2116 men	535	403	292	11	1241
Consumption	and 2544	(43%)	(32%)	(24%)	(1%)	
Survey 1997-	women,	465	306	167	9	979
1998‡	19-97 y	(330-658)	(190-479)	(126-219)	(4-14)	(696-1356)
POLIEP-study	282 men	488	362	191	9	1046
1997-2002‡	and 355	(47%)	(35%)	(18%)	(1%)	
	women,	469	338	185	8	1008
	19-75 y	(375-586)	(251-453)	(147-222)	(4-12)	(798-1261)
Zutphen Elderly	570 men,	494	374	158	12	1037
Study	64-84 y	(48%)	(36%)	(15%)	(1%)	
1985-1995‡		476	333	152	11	977
		(376-582)	(229-450)	(124-181)	(7-15)	(771-1212)

*Values are mean (% contribution) and median (1st quartile-3rd quartile). †All three studies were carried out in the Netherlands. ‡Years of dietary data collection.

adjusted rate ratios (95% CI) were 0.72 (0.53-0.98) for CHD, 0.83 (0.69-1.00) for CVD, 0.81 (0.65-1.00) for cancer and 0.86 (0.76-0.97) for all-cause mortality.

Methodological considerations

Estimation of lignan intake

We have estimated lignan intake in three different populations in the Netherlands. In each of these studies a different method was used to estimate the food consumption. In the National Food Consumption Survey a two-day dietary record method was used, in the POLIEP-Study food consumption was measured using a food frequency questionnaire referring to the year previous to colonoscopy, and in the Zutphen Elderly Study food consumption in the previous month was estimated using a cross-check dietary history method. Nonetheless, in these three different populations, the estimated median intake was comparable.

The contribution to the total lignan intake of SECO and MAT, the two lignans frequently reported in the literature, was only about 25%, whereas the newly discovered enterolignan precursors LARI and PINO contributed about 75% to the total lignan intake (TABLE 7.2). Thus inclusion of LARI and PINO in the lignan database was an important step to be able to measure the exposure to enterolignans.

It is important to note some methodological issues related to the estimation of lignan intake. In all three studies (TABLE 7.2) food consumption data were collected without the predefined aim to determine lignan intake. As a consequence, the dietary surveys did not collect detailed information on the consumption of certain foods, such as oilseeds, that are particularly rich sources of lignans. For example, no distinction was made between wholegrain bread and multigrain bread. Almost all commercially available multigrain breads contain flaxseed, and thus the lignan content of multigrain bread is much higher than that of wholegrain bread. One slice of multigrain bread already may contribute more than 2 mg to lignan intake, whereas our estimation of the habitual lignan intake in the Netherlands was only about 1 mg/day.

The average consumption of multigrain bread has increased in recent years. Before 1990 the market share of multigrain bread was negligible, whereas in 2004 this had already increased to 15%.[1] In addition to bread, flaxseed is also used as an ingredient in granola (muesli), (health)bars, and biscuits, and these sources have also not been queried in detail in the dietary surveys. Thus, especially in more recent epidemiological studies, this may have led to misclassification of the exposure to dietary lignans and to bias in the results. In the Zutphen Elderly Study the food data were collected between 1985 and 1995, so we do not expect that this was an important issue, because the diet of elderly men probably is likely to be to be relatively stable. However, in the National Food Consumption Survey, and in the POLIEP-study in which the food consumption data were collected in 1997 and 1998 and between 1997 and 2002 respectively, and which both included persons of 19 years and older, the misclassification may have been more severe.

An advantage of our studies is that we used a lignan database of Dutch foods that were processed and prepared as usual, except for vegetables that were mostly analyzed raw. However, lignans are relatively heat-stable, and thus it is unlikely that they are lost during household preparation.[2,3]

Bioavailability of lignans

To be absorbed into the body, lignans have to be released from the food matrix. In addition, they have to be released from the glycosides and oligomers, if they are not present as aglycones. For the use in epidemiological studies, the amount of lignans extracted by the analytical method should ideally correspond to the amount of lignans released in the human body. In our analytical method the lignan yield from solid foods was substantially enhanced by the inclusion of alkaline hydrolysis, because it enables the

release of ester-linked lignans. At this time it is not known whether these bound lignans can be liberated in the human body, and whether they are present in the diet in substantial amounts. In flaxseed the major lignan (SECO diglucoside) is bound to an oligomeric structure via ester bonds.[4] Intervention studies have shown that SECO from flaxseed is largely bioavailable.[5] In addition, it has been reported that lignans incorporated into lignins were bioavailable in rats.[6] This shows that at least some of the bound lignans are bioavailable. Food processing may enhance the release of lignans from the food matrix. For instance, milling or crushing of flaxseed substantially improved the bioavailability of lignans.[5]

The intestinal microflora plays an important role in the bioavailability of lignans, as has been shown by the decrease in enterolignan concentrations after use of antibiotics.[7] The relatively large intra- and interindividual variation in plasma enterolignan concentrations can not be explained by differences in the dietary lignan intake alone. Differences in the composition of the intestinal microflora also play an important role. Therefore it is not surprising that we found only a modest correlation between dietary lignan intake and plasma enterolignans in the POLIEP-study.

If further data on the bioavailability of lignans become available they may be used to improve the assessment of exposure to enterolignans. For example, it should be investigated whether the *in vitro* degree of conversion of the plant lignans reflects the *in vivo* situation. First, the *in vitro* experiments need to be repeated with fecal samples from several persons, taken at different times, because so far they have only been determined in a single experiment (TABLE 7.3). If it is possible to get reproducible conversion factors *in vitro*, they could be used to evaluate whether the correlation between dietary lignan intake and plasma enterolignans increases when these conversion factors have been taken into account. Finally, these results need to be confirmed in human intervention trials.

Other enterolignan precursors

We did not include all known enterolignan precursors in our studies. In TABLE 7.3 an overview of the *in vitro* conversion degree of plant lignans is presented. In addition to LARI and PINO, also syringaresinol, 7-hydroxy-MAT, arctigenin, medioresinol and sesamin were identified as new enterolignan precursors.[8-10] However, they had relatively low degrees of conversion. Thus, it is not likely that they are important for the exposure to enterolignans unless they are very abundant in the diet. Recently some data about the presence of these precursors in cereals, oilseeds, nuts, and vegetables became

TABLE 7.3 *In vitro* degree of conversion of plant lignans to enterolignans.

Compound	% Conversion	Reference
LARI	100	8
PINO*	55	8
SECO	72	8
MAT	62	8
syringaresinol*	4	8
arctigenin*	6	8
medioresinol	+†	9
7-hydroxy-MAT	15	8
isolariciresinol	0	8
sesamin	1	10

*Tested as glucosides. †The degree of conversion was not reported

available.[11,12] Syringaresinol, medioresinol and 7-hydroxy-MAT were present in cereals[11,12] and a few vegetables[11] in concentrations comparable to those of the other enterolignan precurors.[12] 7-hydoxy-MAT was the most abundant lignan in wheat and a few other cereals[12] and thus this may be quantitatively important. Arctigenin was a minor component in grain products and nuts.[12] Sesamin was identified only in sesame seeds and almonds.[12] When sesamin is included, sesame seeds had a similar lignan content as flaxseed.[12,13] In an intervention study in women, the plasma enterolignan concentration after consumption of 25 g sesame seeds was comparable to that after consumption of the same amount of flaxseed.[14] This can only be explained by the high amount of sesamin in sesame seeds, and thus it seems that the *in vitro* degree of conversion (only 1%) of sesamin does not reflect the *in vivo* situation. It also indicates that sesame seeds are an important lignan source, and should also be quantified in detail in dietary surveys of lignan intake.

An other potential source of enterolignan exposure that we did not take into account is milk.[15] However, in a Swedish population, the contribution of enterolignans from dairy products to total lignan intake was only 0.3%, so milk does not seem to be an important lignan source.[16]

Absorption of plant lignans without conversion to enterolignans

In our work, we have assumed that in order to be physiologically active, plant lignans have to be converted to enterolignans. However, plant lignans have been identified in human plasma and urine, which shows that they may also be absorbed as such.[17-19] It is supposed that the concentrations of plant lignans are lower than those of the enterolignans, but data are still limited. In four females and two men, consuming their usual Finnish diet, the concentration of MAT in urine was much lower than that of enterodiol and entero-

lactone.[20] In 10 Finnish subjects consuming their habitual diet, the sum of plant lignan concentrations (ISO-LARI, LARI, PINO, SECO, MAT and syringaresinol) in urine was approximately 50-90% lower than that of the enterolignans (END plus ENL).[19] Smeds *et al.* reported serum concentrations of plant lignans and enterolignans in five men and five women consuming their habitual Finnish diet.[21] In all five men, the plant lignans concentrations were lower than those of the enterolignans. In fact, in serum of only one of the men a plant lignan (MAT) could be detected. In three of the five women, concentrations of the plant lignans were higher than those of the enterolignans, mainly due to LARI. Penalvo *et al.* showed that concentrations of plant lignans, particularly PINO, were relatively high in four volunteers shortly after consumption of sesame seeds.

Data on the biological activity of plant lignans as such are also very limited. Thus at this moment, it is not possible to evaluate their relevance *in vivo*. Nonetheless, absorption of the plant lignans may provide an explanation for the observed inverse association between intake of MAT and mortality, although total lignan intake was not related.

Biomarker approach versus assessment of dietary intake

An alternative way to measure lignan exposure is the measurement of enterolignans in biological fluids, such as blood or urine. Advantages of this method are that it takes into account bioavailability, it also includes unknown enterolignan sources, *e.g.* unknown lignan precursors and unknown food sources, and it is not dependent on memory Thus, the use of these biomarkers might give a better representation of the exposure to enterolignans than the quantification of enterolignan precursors in the diet. However, a prerequisite for the biomarker approach is that lignan concentrations reflect long-term intake, which is not necessarily the case. It is assumed that steady-state plasma concentrations of enterolignans can be achieved because they are eliminated slowly (elimination half life 4-13 h) and lignans are present in many foods, and consumed several times per day.[22,23] However, enterolignans will be adequate biomarkers only if persons have a relatively stable dietary pattern. In addition, enterolignan concentrations decrease dramatically after use of antibiotics. Thus, at that time they certainly do not reflect long-term enterolignan exposure. This is an important limitation, since antibiotic use is relatively common and it may take up to one year before enterolignan concentrations are fully recovered.[7]

Also in the biomarker approach, the exposure assessment may be improved by inclusion of additional compounds. Besides END and ENL, a

few other lignan metabolites such as enterofuran and 7-hydroxy-ENL have been identified.[24] In addition, plant lignans that are absorbed as such should also be measured.

Although we think that the biomarker approach is a useful alternative to assess exposure in epidemiological studies, this does not imply that it is not necessary to assess the dietary intake. In order to interpret the results of epidemiological studies it is important to have an idea of the dietary sources, because this also gives an indication of potential confounders. Comparing the concentrations of a biomarker with dietary lignan intake may help to identify potential problems with the exposure assessment. Thus, more studies should evaluate the relative validity of dietary intake and biomarkers. Finally, knowledge about the dietary sources is needed to give dietary recommendations.

Interpretation of findings

In order to compare our findings with those of others, and to gain insight in the state of the art in this field, we have reviewed published data on lignan intake in several populations.

Lignan databases

The first data on the lignan content in a range of foods were published by Thompson *et al.*[25] (TABLE 7.4). They measured the production of enterodiol and enterolactone after *in vitro* fermentation of 68 plant foods, and used this to estimate the lignan content of foods.

All other published lignan databases report lignan contents that were directly measured in foods. Except for the more recent databases they only include data on the lignans SECO and MAT, and in many cases they also contain data on other compounds such as isoflavones or flavonoids. Adlercreutz, Mazur and co-workers have measured SECO and MAT in a variety of Finnish plant foods. They used both acid and enzymatic hydrolysis to release phytoestrogens from plant foods, and they have quantified them using isotope dilution GC-MS.[26] Nurmi *et al.*[27] have later adapted this method for HPLC with coulometric detection, and these data have been included in the database published by Valsta *et al.*[28] In addition, Horn-Ross *et al.* have quantified SECO and MAT in foods consumed in the USA using HPLC-MS.[29] Lignans were extracted with 80% aqueous methanol, and hydrolyzed glycosides with β-glucosidase. Thompson *et al.* have recently published a database that also contains data on PINO and LARI besides SECO and MAT.[30] These lignan contents were determined in Canadian foods using GC-MS. Lignans were

extracted with 70% aqueous methanol, and lignans were released using alkaline hydrolysis and β-glucuronidase.

A comparison of lignan data from various sources is difficult, because differences may arise due to differences in varieties of food, growth conditions, and food processing. In addition, comparison with the data of Mazur *et al.* is complicated because these data are reported on a dry weight basis. However, as far as a comparison of lignan data is possible, it seems that our values for SECO and MAT are comparable or slightly lower than those of Mazur *et al.* and comparable or higher than those of Horn-Ross *et al.* The method of Horn-Ross *et al.* had a relatively high limit of detection (25 μg/100 g), and thus for many foods they found lignan values below the detection limit. In addition, they did not use alkaline or acid extraction, and thus bound lignans may not be released and quantified with their analytical method.

Thompson *et al.* have recently also quantified the four major lignans in foods.[30] In general their values, particularly those of fruits and vegetables, were lower than our lignan values. Their method of lignan extraction and hydrolysis was similar to ours, but because they used GC-MS a much more extensive sample cleanup including several solid phase extractions was necessary. This may have led to losses during the sample preparation and thus to lower lignan values. They did not correct for these potential losses using internal standards, and they did not report the recovery of their method. However, it seems likely that a difference in recovery of the analytical methods may at least partly explain the differences in the lignan contents. The ranking of foods according to their lignan concentration, and the ranking of individual lignans within foods, reported by Thompson *et al.* was generally in agreement with our results. Thompson *et al.* have also reported that the ranking of foods within food groups according to their lignan contents was similar to the ranking of foods according to their production of enterolignans in their earlier *in vitro* method. This provides some reassurance that the four lignans measured indeed are major enterolignan precursors in foods.

The other lignan databases in TABLE 7.4 are compilations of published lignan data. Thus, a drawback of these databases is that lignan contents have been measured with various analytical methods. In addition, the food samples used, and the methods of preparation, may not be the same as those in the target population. All of the literature compilations include lignan contents published by Mazur *et al.,* who mostly reported data for unprocessed foods on a dry weight basis. So the lignan contents of the foods as they are actually consumed had to be estimated from these lignan values, which is less reliable than actually measuring lignan contents in these foods.

TABLE 7.4 Overview of published lignan databases for the use in epidemiological studies.

Reference	Lignan data	Components	Remarks
Thompson (1991)[25]	Thompson[25]	END, ENL*	– Production of enterodiol and enterolactone from foods after *in vitro* fermentation with fecal microflora – Not developed for epidemiological studies
USDA (1998)[43]	Mazur[26,44,45]	SECO, MAT	– Database primarily aimed at isoflavones; data on SECO and MAT not comprehensive.
Pillow (1999)[46]	Mazur,[26,44,45,47] Obermeyer,[48] Thompson[25]	SECO, MAT, END, ENL*, isoflavones, coumestrol, flavonoids, phytosterols	
Horn-Ross (2000)[29]	Horn-Ross[29]	SECO, MAT, isoflavones, coumestrol	– Method of preparation of foods as usual in the population – Relatively high detection limit (25 µg/100g)
De Kleijn (2001)[40]	Mazur,[47,49,50] unpublished data, USDA,[43] Thompson[25]	SECO, MAT, isoflavones, coumestrol	– Phytoestrogen score based on highest value reported in literature, instead of actual concentrations – Thompson data used to estimate SECO and MAT
Valsta (2003)[28]	Mazur,[26,44,45,47,49,50] Nurmi,[27] previously unpublished data	SECO, MAT, isoflavones	– Have made a quality assessment of the incorporated data
Linseisen (2004)[32]	Mazur,[26,47,49-51] Horn-Ross,[29] Thompson[25]	SECO, MAT, END, ENL*, isoflavones, coumestrol	
Bhakta (2005)[52]	Mazur,[44,47,50] unpublished data	SECO, MAT, isoflavones	– Have made a quality assessment of incorporated data – Have estimated the relative validity of lignan intake compared to plasma ENL – Have incorporated new data on food items relevant for their population

TABLE 7.4 Continued

Reference	Lignan data	Components	Remarks
Schabath (2005)[36]	Pillow,[46] Horn-Ross,[29] De Kleijn,[40] Valsta[28]	SECO, MAT, END, ENL*, isoflavones, phytosterols	– Have erroneously summed up plant lignans with enterolignans estimated after *in vitro* fermentation
Milder (2005)[53]	Milder[53]	SECO, MAT, LARI, PINO	– Method of preparation of foods as usual in the population – Have estimated the relative validity of lignan intake compared to plasma END and ENL
Hedelin (2006)[16]	Mazur,[50,54,55] Valsta,[28] unpublished data	SECO, MAT, LARI, PINO, syringaresinol, medioresinol, END, ENL†	– LARI and PINO, syringaresinol, and medioresinol only for bread and cereal products. – END and ENL were measured in milk; lignan contents in dairy products were estimated using these values.
Fink (2006)[37]	Reinli and Block,[56] Pillow,[46] USDA,[57] De Kleijn[40], unpublished data	SECO, MAT, END, ENL*, isoflavones, flavonoids	
Thompson (2006)[30]	Thompson[30]	SECO, MAT, PINO, LARI, isoflavones, coumestrol	– Method of preparation of foods as usual in the population
Blitz (2007)[58]	Mazur,[26,44,45,49-51] De Kleijn,[40] Pillow,[46] Valsta,[28] Meagher and Beecher,[59] Horn-Ross[29]	SECO, MAT	

*Production of END and ENL from foods after *in vitro* fermentation with fecal microflora. †END and ENL determined in foods.

TABLE 7.5 Median daily lignan intakes in several populations estimated using the enterolignan production from foods.*

Reference	Food survey	Design (outcome)	Population	END (µg/d)†	ENL (µg/d)†	Total (µg/d)†
McCann (2002)[60]	FFQ Block	CC (breast cancer)	617 pre-MP F, USA	-	-	565 (260-1575)‡
			933 post-MP F, USA	-	-	595 (315-1400)‡
Walcott (2002)[31]	FFQ Block	CC (testicular cancer)	136 M, USA, controls	-	-	223 (170-302)§
Linseisen (2004)[32]	FFQ EPIC	CC (breast cancer)	666 pre-MP F, Germany, controls	376 (242-582)	331 (227-453)	752 (483-1164)
			278 pre-MP F, Germany, cases	363 (235-555)	316 (227-446)	726 (470-1110)
Keinan-Boker (2004)[61]	FFQ EPIC	CO (breast cancer)	280 F, Netherlands	400 (310-500)	270 (210-330)	670 (530-830)
Schabath (2005)[36]	FFQ EPIC	CC (lung cancer)	1674 M+F, USA, controls	-	-	354 (252-478)
			774 F, USA, controls	-	-	336 (242-459)
			900 M, USA, controls	-	-	371 (261-499)
Touillaud (2005)[35]	FFQ Block	CA (breast cancer)	88 F, USA, cases, ER(+)	92 (63-130)	128 (79-260)	224 (142-332)
			36 F, USA cases, ER(-)	109 (82-144)	154 (103-241)	265 (190-407)
Piller (2006)[62]	FFQ Block	CC (breast cancer)	573 pre-MP F, Germany, controls	382 (242-578)	332 (223-453)	-
			267 pre-MP F, Germany, cases	365 (235-553)	317 (227-445)	-

*All studies used the lignan database published by Thompson *et al.*[25] Abbreviations: -, not reported; FFQ, food frequency questionnaire; EPIC, European Prospective Investigation into Cancer and Nutrition; CC case-control; CO, cohort; CA, case-case; F, female; M, male; pre-MP, premenopausal; post-MP postmenopausal; ER(+), estrogen receptor positive; ER(-), estrogen receptor negative. Note that the molecular weight of the plant lignans (358-362 g/mol) is somewhat higher than that of enterodiol (302 g/mol) and enterolactone (298 g/mol), thus the corresponding amount of dietary precursors is somewhat higher. †Values are median (25^{th} percentile-75^{th} percentile), unless otherwise indicated. ‡Midpoint of 2^{nd} tertile (midpoint of 1^{st} tertile–midpoint of 3^{rd} tertile). §Lignan intake per 1000 kcal.

Estimation of lignan intake

Almost all previous studies on lignan intake have only included SECO and MAT, and thus these studies have severely underestimated the intake of enterolignan precursors. In a few studies an alternative method to quantify lignan intake was used. In these studies, the *in vitro* enterolignan production, as reported by Thompson *et al.*[25] was used to estimate the lignan intake (TABLE 7.5). The estimated median lignan intake ranged from 223 µg/d in American men[31] to more than 700 µg/d in German women.[32] However, the aim of the study of Thompson *et al.* was to screen foods on the presence of enterolignan precursors, rather than to develop a database for epidemiological studies. Thus in our opinion these estimates of lignan intake are not very reliable.

TABLE 7.6 presents an overview of studies that have reported the intake of SECO and MAT. Most studies have reported the median intake; therefore we choose this measure to compare the lignan intake among studies. However, some studies have only reported the (arithmetic) mean. Because lignan intake is generally skewed towards higher values, the mean is generally higher than the median. The estimates of lignan intake ranged from 108 µg/d (mean)[33] to 6620 µg/d (median).[34] In all studies, the intake of MAT was substantially lower than that of SECO.

In general, the estimates obtained with the same database are closer to each other than those obtained with different databases. For example, in studies that used the database published by Horn-Ross, lignan intake ranged from 108 to 177 µg/d, and for studies that used the database of De Kleijn from 472 to 1630 µg/d. The highest median lignan intakes have been obtained in a few recent American studies that have incorporated data of the Pillow/USDA database.[34-37] It is remarkable that in these studies 80 to 99%[36,37] of the lignan intake was from tea and coffee, whereas in other studies the contribution of tea and coffee to lignan intake was much lower. In the Pillow database, data for the lignan concentrations of tea and coffee were derived from those published by Mazur *et al.*[38] Mazur *et al.* have reported lignan contents of tea *leaves* and coffee *powder*, on dry weight basis. Thus, the amount of lignans in brewed coffee and tea infusion had to be estimated using these data. Probably this has led to overestimation of the lignan content of prepared tea and coffee. In the study by Strom *et al.*,[39] in which the Pillow database was also used, the reported lignan intakes were much lower, but they used an older version of this database. Finally, because most databases have only been used within one country, it is difficult to compare lignan intakes among countries.

TABLE 7.6 Estimated daily intake of SECO and MAT in several populations.

Reference	Database	Food survey	Design (outcome)	Population	SECO (μg/d)*	MAT (μg/d)*	Total (μg/d)*
Strom (1999)[39]	Pillow	FFQ Block	CC (prostate cancer)	83 M, USA, cases	819‡	57‡	-
				107 M, USA, controls	483‡	46‡	-
Walcott (2002)[31]	Pillow	FFQ Block	CC (testicular cancer)	136 M, USA, controls			698 (275-1416)
Touillaud (2005)[35]	Pillow	FFQ Block	CA (breast cancer)	88 F, USA, cases, ER+	3121 (1255-5100)	28 (12-172)	3159 (1284-5301)
				36 F, USA, cases, ER(-)	2559 (1409-5090)	67 (14-170)	2702 (1416-5256)
Horn-Ross (2000)[63]	Horn-Ross	FFQ Block	CO	447 F, 50-79 y, USA	139§	36§	175§
Horn-Ross (2001)[64]	Horn-Ross	FFQ Block	CC (breast cancer)	1610 F, USA, controls	122 (75-175)	30 (18-50)	158 (104-159)
Horn-Ross (2002)[65]	Horn-Ross	FFQ Block	CC (thyroid cancer)	1134 F, USA	70 (51-94)$	34 (24-48)$	-
Horn-Ross (2002)[33]	Horn-Ross	FFQ Block	CO (breast cancer)	111526 F, 21-103 y, USA	85§	23§	108§
Horn-Ross (2003)[66]	Horn-Ross	FFQ Block	CC (endometrial cancer)	470 F, USA, controls	138 (87-197)	30 (18-49)	177 (121-239)
				482 F, USA, cases	127 (85-176)	32 (20-51)	162 (115-228)
Horn-Ross (2006)[67]	Horn-Ross	FFQ Block	CO	195 F, USA, pre-study	110‡	23‡	137‡
				195 F, USA, post-study	115‡	25‡	137‡
		24-h recall	CO	195 F, USA	133‡	21‡	161‡
De Kleijn (2001)[40]	De Kleijn	FFQ Willett	CS (CVD risk factors)	964 Post-MP F, USA	560 (399-778)	19 (12-28)	578 (416-796)
				939 Post-MP F, USA	534 (383-761)	25 (16-36)	579 (416-798)
Keinan-Boker (2002)[41]	De Kleijn	FFQ EPIC	CS	17357 F, 49-70 y, The Netherlands	988 (676-1285)	73 (43-106)	1110§
McCann (2003)[68]	De Kleijn	FFQ Block	CC (ovarian cancer)	696 F, USA, controls	-	-	525 ± 288
				124 F, USA, cases	-	-	477 ± 290
Van der Schouw (2002)[69]	De Kleijn	FFQ EPIC	CS (aortic stiffness)	403 Post-MP F, The Netherlands	-	-	630 (330-870)
McCann (2004)[70]	De Kleijn	FFQ Block	CC (breast cancer)	593 Pre-MP F, USA, controls	-	-	472 (329-673)
				315 Pre-MP F, USA, cases	-	-	510 ± 18†
				1443 Post-MP F, USA, controls	-	-	504 (337-713)
				807 Post-MP F, USA, cases	-	-	576 ± 11

TABLE 7.6 Continued

Reference	Database	Food survey	Design (outcome)	Population	SECO (µg/d)*	MAT (µg/d)*	Total (µg/d)*
Kreijkamp-Kaspers (2004)[71]	De Kleijn	FFQ EPIC	CS (CVD risk factors)	301 Post-MP F, 60-75 y, Post-MP, The Netherlands	-	-	1630‡#
Van der Schouw (2005)[72]	De Kleijn	FFQ Rimm	CS (CVD risk factors)	468 M, 47-83 y, USA	670 (450-920)	30 (20-50)	690 (490-930)
Van der Schouw (2005)[42]	De Kleijn	FFQ EPIC	CO (CVD)	16165 F, 49-70 y, The Netherlands	992 (681-1280)	74 (43-106)	1081 (738-1392)
Franco (2005)[73]	De Kleijn	FFQ EPIC	CS (cognitive function)	394 Post-MP F The Netherlands	-	-	620 (320-860)
McCann (2005)[74]	De Kleijn	FFQ Block	CC (prostate cancer)	538 M, USA, controls	-	-	446 (335-604)
				433 M, USA, cases	-	-	
McCann (2006)[75]	De Kleijn	FFQ Block	CC (breast cancer)	593 Pre-MP F, USA, controls	-	-	472 (329-673)
				1443 Post-MP F, USA, controls	-	-	504 (337-713)
Kilkkinen (2003)[76]	Valsta	24 h recall	CS	1359 M, Finland	126 ± 3†	46 ± 53†	173 ± 3†
				1493 F, Finland	121 ± 1†	30 ± 1†	151 ± 3†
Valsta (2003)[28]	Valsta	24 h recall	CS	2862 M and F, Finland	396 ± 1571	38 ± 18	434 ± 1575
				1361 M, Finland	240 ± 9387	45 ± 20	285 ± 9347
				1501 F, Finland	570 ± 1665	31 ± 13	601 ± 1670
Linseisen (2004)[32]	Linseisen	FFQ EPIC	CC (breast cancer)	666 Pre-MP F Germany, controls	529 (274-1280)	29 (20-39)	563 ± 1331
				278 Pre-MP F Germany, cases	531 (275-1409)	28 (20-38)	570 ± 1428
Bhakta (2005)[52]	Bhakta	9-12 monthly 24 h recalls	CC (breast cancer)	108 F, UK	101 (73-178)	8 (5-12)	110 (78-191)
		FFQ Sevak		108 F, UK	142 (81-327)	9 (6-18)	149 (87-346)

TABLE 7.6 Continued

Reference	Database	Food survey	Design (outcome)	Population	SECO (μg/d)*	MAT (μg/d)*	Total (μg/d)*
Dos Santos Silva (2004)[77]	Bhakta	FFQ Sevak	CC (breast cancer)	477 South Asian F, UK, controls	122 (80-225)	8 (5-13)	129 (85-236)
				240 South Asian F, UK, cases	124‡	7‡	130‡
Schabath (2005)[36]	Schabath	FFQ Block	CC (lung cancer)	1674 M+F			5358 (3413-9116)
				774 F			4964 (2941-8402)
				900 M			5958 (3673-9697)
Hedelin (2006)[16]	Hedelin	FFQ Chang	CC (prostate cancer)	1130 M, Sweden, controls	125‡	42‡	-
				1499 M, Sweden, cases	129‡	43‡	-
Fink (2006)[34]	Fink	FFQ Block	CC (breast cancer)	487 Pre-MP F controls	-	-	5920‡
				457 Pre-MP F cases	-	-	5970‡
				953 Post-MP F controls	-	-	6620‡
				977 Post-MP F cases	-	-	6010‡
Fink (2006)[37]	Fink	FFQ Block	CS (breast cancer)	1500 F, 20-95 y, USA, controls	-	-	4840‡
Cotterchio (2006)[79]	Blitz	FFQ Hawaii	CC (colorectal cancer)	1890 M+F, 20-74 y, Canada	-	-	207‡

*Values are median (25^{th} percentile-75^{th} percentile) or mean ± SD, unless otherwise indicated; Abbreviations:-not reported; FFQ, food frequency questionnaire; EPIC, European Prospective Investigation into Cancer and Nutrition; CO, cohort; CS, cross-sectional; CC, case-control; CA, case-case; F, female; M, male; pre-MP, pre-menopausal; post-MP postmenopausal; ER(+), estrogen receptor positive; ER(-), estrogen receptor negative. ‡Median. §Mean. †Mean ± SEM. $Midpoint of 2^{nd} tertile (midpoint of 1^{st} tertile–midpoint of 3^{rd} tertile). #Energy-adjusted.

Lignan intakes in the Netherlands. In all three studies described in this thesis, the median total lignan intake was approximately 1 mg per day (TABLE 7.2). For SECO plus MAT this was approximately 150-200 µg/d. Previous estimates of the intake of SECO plus MAT in the Netherlands were based on the database published by the Kleijn *et al.*[40] In postmenopausal women aged 49-70 y, the median intake of SECO and MAT alone was already 1 mg/day,[41,42] thus 5-6 times higher than in our studies. In part this may be explained by the difference in study population, because we found that lignan intake increased with age (CHAPTER 4). However, we think that they have overestimated the lignan intake, because they used category scores based on the highest reported value in literature, instead of the actual lignan concentrations. In addition, for some products, the amount of SECO and MAT was derived from the amount of enterolignans produced after *in vitro* fermentation. This leads to an overestimation of the amount of SECO and MAT if the product also contains other enterolignan precursors.

Relation of lignan intake with cardiovascular diseases and cancer

In CHAPTER 1 we have summarized the biological activities of lignans, and the evidence for their protective effects on cancer and cardiovascular diseases from animal and epidemiological studies. *In vitro* and animal studies provided several mechanisms by which lignans could protect against both cardiovascular diseases and cancer. Epidemiological studies yielded conflicting results, which could mainly be attributed to the lack of comprehensive data on the lignan content of foods. Another concern related to these data was that significant lower risks were found in case-control studies, but not in prospective studies. After diagnosis of a serious disease, patients may change their diet and/or the recall of their previous diet. This may lead to spurious associations, and therefore it is important that these results are confirmed in prospective studies.

In the prospective Zutphen Elderly Study, we did not find a significant association between total lignan intake and all-cause or disease-specific mortality, after adjustment for potential confounders. However, we found a significant inverse association of intake of MAT with coronary heart disease, cardiovascular diseases and all-cause mortality, and borderline significant with cancer mortality. This may be explained by the fact that plant lignans can also be absorbed without conversion to enterolignans, and thus they may have differential effects in the body. Few previous studies have investigated the role of individual lignans. In a German case-control study breast cancer risk was inversely associated with intake of MAT RR: 0.58, 95% CI: 0.37-0.94, but not with intake of SECO or total lignan intake.[32]

As always in observational studies, residual confounding should be considered as a potential explanation for the observed associations between intake of MAT and mortality. The association between the intake of MAT and chd mortality observed in the Zutphen Elderly Study was attenuated after adjustment for wine consumption. This could not be attributed to alcohol, because adjustment for alcohol did not change the strength of the association. Perhaps, bioactive compounds other than alcohol may (partly) explain the inverse association found for MAT.

Conclusions and recommendations for future research

Inclusion of LARI and PINO in the assessment of lignan intake was an important improvement in the estimation of exposure to enterolignan precursors. Addition of more enterolignan precursors may further improve exposure assessment in epidemiological studies. Food consumption surveys specifically focusing on the quantification of lignan intake should be carried out. At least the consumption of oilseeds such as flaxseed and sesame seed should be quantified in detail. Although we have substantially improved the estimation of the lignan intake compared to previous studies, we still found a moderate correlation (r_s = 0.18) between plasma and dietary lignans in the POLIEP-study. The correlation between plasma and dietary lignans should be re-evaluated using lignan intake data of the suggested specific food consumption surveys. Plasma END concentrations were determined by consumption of lignan-rich foods, but also by age and previous use of antibiotics. Plasma ENL concentrations were determined by consumption of lignan-rich foods, weight, current smoking and frequency of defecation. Even when we took the non-dietary determinants into account, we could only explain a small part of the variance in plasma enterolignan concentrations. Thus, in addition to improved dietary data, more insight in other factors influencing the plasma lignan concentration, in particular the role of the intestinal microflora is needed.

In the Zutphen Elderly Study we did not find an association between total lignan intake and mortality, whereas the intake of MAT was inversely associated with CHD, CVD, cancer and all-cause mortality. This may perhaps be explained by the absorption of MAT without conversion to enterolignans. Our study was the first to investigate the association between intake of four individual lignans and mortality. Thus, before conclusions can be drawn, these results need to be confirmed in other prospective studies.

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Summary

Plant lignans are diphenolic compounds, comprised of two phenylpropanoid (C6-C3) units. They can be found in a wide variety of plant foods. After ingestion, some plant lignans can be converted to the enterolignans, enterodiol (END) and enterolactone (ENL), by the intestinal microflora, and absorbed into the body. Only a minor part of dietary lignans is absorbed as such. For about two decades only secoisolariciresinol (SECO) and matairesinol (MAT) were considered enterolignan precursors, but recently several new precursors have been identified, of which especially lariciresinol (LARI) and pinoresinol (PINO) have a high degree of conversion. Lignans possess several biological activities, such as antioxidant and estrogen-like activities by which they may reduce the risk of cardiovascular diseases and cancer. In epidemiological studies some evidence for a protective effect of lignans against hormone-related cancers and cardiovascular diseases was found, but the results were inconsistent, probably due to a lack of comprehensive data on the lignan contents of foods and diets.

The main aims of the project described in this thesis were to determine the lignan content of Dutch plant foods, to identify major dietary sources of lignans, and to evaluate the associations of lignan intake with plasma enterolignans, and with cardiovascular, cancer, and all-cause mortality.

First we developed a liquid chromatography-mass spectrometry (LC-MS/MS) method for the quantification of LARI, PINO, SECO and MAT in foods and beverages (CHAPTER 2). Lignans were liberated from the food matrix of solid foods using alkaline methanolic extraction. To release lignans from glycosides, the obtained extract, or pure beverage was enzymatically hydrolyzed using *Helix pomatia* β-glucuronidase/sulfatase. After ether extraction lignans were quantified against d_6-MAT and d_8-SECO. The method was optimized using four model products: broccoli, bread, flaxseed and tea. Detection limits were 4-10 µg/100 g dry weight for solid foods, and 0.2-0.4 µg/100 mL for beverages. Within-run and between-run coefficients of variation were 6-21% and 6-33% respectively. The recovery of lignans added to model products was satisfactory (73-123%), except for MAT added to bread (51-55%). The method is suitable for the use in a wide range of foods and beverages.

Subsequently, we constructed a database with lignan contents of 83 solid foods and 26 beverages commonly consumed in the Netherlands (CHAPTER 3). Flaxseed (≈300 mg/100 g) and sesame seeds (≈40 mg/100 g) were by far the most abundant lignan sources. Flaxseed contains mainly SECO, and sesame seed mainly PINO. Lignan concentrations for grain products, vegetables, fruits and legumes varied mostly between 50 and 200 μg/100 g. Higher concentrations were found for breads with sesame or flaxseed (7-12 mg/100 g), most *Brassica* vegetables (185-2321 μg/100 g), garlic (536 μg/100 g), French beans (273 μg/100 g), apricots (450 μg/100 g), strawberries (334 μg/100 g), and peaches (293 μg/100 g). Lignan concentrations in beverages ranged from 0 for cola to 91 μg/100 mL for red wine.

We used this database to estimate lignan intake in a representative sample of 4660 Dutch adults participating in the Dutch Food Consumption Survey, carried out in 1997-1998 (CHAPTER 4). The median total lignan intake was 979 μg/day. Total lignan intake did not differ between men and women, but the lignan density of the diet was significantly ($P < 0.001$) higher in women than in men. Lignan intake was strongly skewed towards higher values (range 43-77 584 μg/day, mean 1241 μg/day). LARI and PINO contributed 75% to lignan intake, whereas SECO and MAT contributed only 25%. Remarkably, the major food sources of lignans were beverages (37%), followed by vegetables (24%), nuts and seeds (14%), bread (9%) and fruits (7%). Lignan intake was significantly ($P < 0.001$) correlated with intake of dietary fiber ($r_s = 0.46$), folate ($r_s = 0.39$) and vitamin C ($r_s = 0.44$). Older persons, nonsmokers, vegetarians, and persons with a low BMI, or a high socio-economic status had higher lignan intakes than their counterparts.

Both dietary intake and plasma enterolignans have been used to evaluate exposure to lignans. In order to evaluate the relative validity of these exposure measures, we studied the relationship between plasma and dietary lignan intake in the POLIEP-Study, a case-control study on colorectal adenomas (CHAPTER 5). The population consisted of 637 men and women, aged 19-75 years, who underwent an endoscopy. We excluded participants who used antibiotics in the preceding calendar year. We found modest associations between lignan intake and plasma END ($r_s = 0.09$, $P = 0.03$) and between lignan intake and plasma ENL ($r_s = 0.18$, $P < 0.001$). The correlation of total lignan intake with plasma enterolignans was slightly stronger than that of only SECO plus MAT. The plasma concentrations of both END and ENL were associated with intake of dietary fiber and vegetable protein, but not with intake of other macronutrients. The relation between lignan intake and plasma END was modulated by age and previous use of antibiotics, whereas for

ENL it was modulated by weight, current smoking and frequency of defecation. However, even when we included these non-dietary factors in the regression models the explained variance in plasma END and ENL remained low (2 and 13%, respectively).

We studied the relation between the dietary lignan intake and mortality in the Zutphen Elderly Study (CHAPTER 6). This is a prospective cohort study in which 570 men aged 64-84 y were followed for 15 years. Lignan intake was related to mortality using Cox proportional hazards analysis. The total lignan intake was not related to mortality. However, the intake of MAT was inversely associated with coronary heart disease (CHD), cardiovascular diseases (CVD), cancer, and all-cause mortality. Multivariate adjusted rate ratios (95% CI) per 1-SD increase in intake were 0.72 (0.53-0.98) for CHD, 0.83 (0.69-1.00) for CVD, 0.81 (0.65-1.00) for cancer, and 0.86 (0.76-0.97) for all-cause mortality.

In CHAPTER 7 the main findings presented in this thesis were discussed. There is some evidence that addition of more enterolignan precursors, may improve the exposure assessment in epidemiological studies. Food consumption surveys specifically focusing on the quantification of lignan intake should be carried out. At least, the consumption of oilseeds, such as flaxseed and sesame seed should be quantified in detail. We found a moderate correlation (r_s = 0.18) between plasma and dietary lignans in the POLIEP-study. This correlation should be re-evaluated using lignan intake data of the suggested specific food consumption surveys.

In conclusion, the habitual consumption of dietary lignans in the Netherlands was approximately 1 mg per day. Tea, vegetables, coffee, seeds, bread and fruits were the most important sources of lignans in the Netherlands. We did not find a relation between total lignan intake and mortality. However, intake of MAT was inversely associated with cardiovascular diseases, particularly CHD, and all-cause mortality. Our study was the first to investigate the association between intake of four individual lignans and mortality. Before conclusions can be drawn, these results need to be confirmed in other prospective studies.

Samenvatting

Lignanen zijn stoffen die voorkomen in veel plantaardige levensmiddelen. De molecuulstructuur van lignanen bestaat altijd uit twee C6-C3 eenheden en ze behoren tot de groep van de polyfenolen. In het lichaam kunnen sommige plantaardige lignanen worden omgezet in enterodiol (END) en enterolacton (ENL) door de darmbacteriën. END en ENL worden ook wel de enterolignanen genoemd. Enterolignanen kunnen goed door het lichaam worden opgenomen, terwijl slechts een klein deel van de plantaardige lignanen als zodanig in het bloed terecht komt. Lange tijd werd aangenomen dat slechts twee van de plantaardige lignanen, secoisolariciresinol (SECO) en matairesinol (MAT) worden omgezet door de darmbacteriën, maar later bleek dit voor meer plantaardige lignanen het geval te zijn. Meestal wordt slechts een klein deel omgezet, maar twee lignanen, lariciresinol (LARI) en pinoresinol (PINO), hebben een vergelijkbare omzettingsgraad als SECO en MAT.

Lignanen beschikken over verschillende biologische functies, zoals antioxidant en oestrogeen-achtige activiteit, waardoor ze de kans op hart en vaatziekten en kanker zouden kunnen verlagen. In sommige epidemiologische studies was een hoge lignaaninname inderdaad geassocieerd met een lager risico op deze ziekten, maar in andere studies werd dit niet gevonden. Een belangrijke reden voor de wisselende resultaten is het gebrek aan afdoende gegevens over lignaangehalte in levensmiddelen en het dieet.

De hoofddoelen van het project beschreven in dit proefschrift waren dan ook het bepalen van het lignaangehalte van plantaardige levensmiddelen in Nederland en het identificeren van de belangrijkste voedingsbronnen van lignanen. Ook hebben we onderzocht in hoeverre de lignaaninname via de voeding samenhangt met de concentratie enterolignanen in het bloed en met sterfte aan hart- en vaatziekten en kanker en totale sterfte.

Eerst hebben we een analysemethode ontwikkeld voor het bepalen van LARI, PINO, SECO en MAT in vaste levenmiddelen en dranken (HOOFDSTUK 2). De eerste stap van de analysemethode is het extraheren van de lignanen uit de voedingsmatrix van vaste stoffen met behulp van 70% methanol/water, dat basisch is gemaakt door de toevoeging van 0.3 M natriumhydroxide

(NaOH). Voor dranken is deze eerste stap niet nodig, maar verder is de methode voor vaste stoffen en dranken gelijk. Lignanen komen vaak voor als glycosiden, dat wil zeggen dat ze zijn verbonden aan een of meer suikergroepen. Deze suikergroepen worden verwijderd door het toevoegen van een enzympreparaat (*Helix pomatia* β-glucuronidase/sulfatase). Vervolgens vindt er nogmaals een extractie plaats met behulp van ether, en worden er standaarden toegevoegd (d_6-MAT and d_8-SECO). De lignanen worden vervolgens gemeten m.b.v. vloeistof chromatografie massaspectometrie (LC-MS).

De methode is geoptimaliseerd met behulp van vier testproducten: broccoli, brood, lijnzaad en thee. De detectielimiet voor vaste levensmiddelen was 4-10 µg/100 g droge stof en voor dranken 0.2-0.4 µg/100 mL. De binnen-dag variatiecoëfficiënt was 6-21% en de tussen-dag variatiecoëfficiënt was 6-33%. De recovery van lignanen die aan de modelproducten werden toegevoegd was toereikend (73-123%), behalve voor MAT toegevoegd aan brood (51-55%). Dus de methode is geschikt voor het bepalen van lignanen in een breed spectrum aan plantaardige producten.

Vervolgens hebben we een voedingsmiddelentabel samengesteld met lignaangehaltes van 83 vaste producten en 26 dranken, die gangbaar zijn in het Nederlandse voedingspatroon (HOOFDSTUK 3). Lijnzaad (≈300 mg/100 g) en sesamzaad (≈40 mg/100 g) hadden veruit de hoogste lignaangehaltes. Lijnzaad bevat vooral SECO en sesamzaad vooral PINO. De lignaangehaltes van graanproducten, groenten, fruit en peulvruchten lagen meestal tussen de 50 en 200 µg/100 g. Producten met hogere gehaltes waren: sesam- en lijnzaad (7-12 mg/100 g), de meeste *Brassica* soorten (185-2321 µg/100 g), knoflook (536 µg/100 g), sperziebonen (273 µg/100 g), abrikozen (450 µg/100 g), aardbeien (334 µg/100 g) en perziken (293 µg/100 g). De lignaangehaltes van dranken varieerden van 0 (voor cola) tot 91 µg/100 g (voor rode wijn).

Met behulp van de voedingsmiddelentabel hebben we de gemiddelde lignaaninname in een representatieve steekproef van de Nederlandse bevolking geschat. Deze steekproef bestond uit 4660 volwassenen die deel namen aan de Nationale Voedselconsumptiepeiling in 1997-1998 (HOOFDSTUK 4). De mediane lignaaninname was 979 µg/dag. De totale lignaaninname verschilde niet tussen mannen en vrouwen, maar in verhouding tot hun energie-inname hadden vrouwen een significant ($P < 0.001$) hogere lignaaninname dan mannen. De lignaaninname was sterk scheef verdeeld naar hogere waarden (range 43-77 584 µg/dag, gemiddelde 1241 µg/dag). De bijdrage van LARI en PINO aan de totale lignaaninname was 75%, voor SECO en MAT was dit slechts 25%.

Het is opvallend dat dranken de belangrijkste bron van lignanen waren (37%). Andere belangrijke bronnen waren groenten (24%), noten en zaden

(14%), brood (9%) en fruit (7%). De lignaaninname was significant ($P < 0.001$) gecorreleerd met de inname van voedingsvezel ($r_s = 0.46$), foliumzuur ($r_s = 0.39$) en vitamine C ($r_s = 0.44$). Oudere personen, niet-rokers, vegetariërs en personen met een lage body mass index (BMI), of hoge socio-economische status, hadden een hogere lignaaninname dan hun tegenhangers.

De correlatie tussen de concentratie enterolignanen in het bloed (plasma) en de inname van lignanen via de voeding hebben we bestudeerd in de POLIEP-studie, een patiëntcontrole onderzoek met betrekking tot darmpoliepen. (HOOFDSTUK 5). De onderzoekspopulatie bestond uit 637 mannen en vrouwen van 19-75 jaar die een endoscopie hebben ondergaan. Het gebruik van antibiotica kan de darmbacteriën die zorgen voor de omzetting van plantaardige lignanen in enterolignanen aantasten. Daarom werden gegevens van personen die in het voorafgaande kalenderjaar antibiotica hadden gebruikt niet meegenomen in de analyses. We vonden een zwakke correlatie tussen de lignaaninname en END concentraties in plasma ($r_s = 0.09$, $P = 0.03$) en een matig sterke correlatie tussen de lignaaninname en de enterolacton concentraties in plasma ($r_s = 0.18$, $P < 0.001$). De correlatie van de totale lignaaninname met de concentraties van enterolignanen in plasma was iets sterker dan die van alleen SECO en MAT. De plasmaconcentratie van zowel END als ENL was gecorreleerd met de inname van voedingsvezel en plantaardig eiwit, maar niet met de inname van andere macronutriënten.

Naast de lignaaninname, waren leeftijd en eerder antibioticagebruik onafhankelijke determinanten van de END concentratie in het plasma. Gewicht, huidig roken, en ontlastingsfrequentie waren determinanten van de ENL concentratie in het plasma. Maar zelfs als deze determinanten in de regressiemodellen werden meegenomen, bleef de verklaarde variantie in END en ENL plasma concentraties laag (2-13%).

Vervolgens hebben we de relatie tussen lignaaninname en sterfte bestudeerd in de Zutphen Ouderen Studie (HOOFDSTUK 6). Dit is een prospectieve cohortstudie waarin 570 mannen van 64-84 jaar 15 jaar lang werden gevolgd. De relatie tussen lignaaninname en sterfte werd geanalyseerd met behulp van Cox proportionele hazards analyse. Er was geen relatie tussen de totale lignaan inname en sterfte. De inname van MAT was wel invers geassocieerd met de sterfte aan coronaire hartziekten (CHZ), hart- en vaatziekten (HVZ) en kanker. De rate ratio's (95% betrouwbaarheidsinterval) per standaard deviatie toename in de lignaaninname waren 0.72 (0.53-0.98) voor CHZ, 0.83 (0.69-1.00) voor HVZ 0.81 (0.65-1.00) voor kanker, en 0.86 (0.76-0.97) voor totale sterfte.

In HOOFDSTUK 7 zijn de belangrijkste resultaten van dit proefschrift samengevat en bediscussieerd. Er zijn aanwijzingen dat het bepalen van nog meer plantaardige precursors van enterolignanen de schatting van de blootstelling in epidemiologische studies zou kunnen verbeteren. Ook zouden voedselconsumptie-onderzoeken speciaal gericht op de lignaaninname moeten worden uitgevoerd. Hierin zou ten minste de consumptie van oliezaden zoals lijnzaad en sesamzaad exact moeten worden bepaald. Wij vonden slechts een matig sterke correlatie tussen de lignaaninname en de concentraties van lignanen in plasma. Deze correlatie dient opnieuw te worden geëvalueerd met gegevens van de bovengenoemde specifieke voedselconsumptie-onderzoeken.

Concluderend kunnen we stellen dat de gebruikelijke lignaaninname in Nederland ongeveer 1 mg per dag is. Thee, groenten, koffie, zaden, brood en fruit zijn de belangrijkste bronnen van lignanen in Nederland. We vonden geen relatie tussen de totale lignaaninname en sterfte. Wel was de inname van MAT invers geassocieerd met sterfte aan hart- en vaatziekten, vooral coronaire hartziekten en totale sterfte. Onze studie was de eerste studie waarin de associatie tussen vier individuele lignanen en sterfte is onderzocht. Daarom moet dit resultaat worden bevestigd in andere prospectieve studies, voordat conclusies kunnen worden getrokken.

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Curriculum vitae

Ivon Elisabeth Josephina Milder was born on 28 January 1976 in Nijmegen, the Netherlands. She completed secondary school (VWO) at the Overbetuwe College in Bemmel in 1994. From 1994 till 1999 she studied Food Technology at Wageningen University. As part of this study, she conducted MSc projects in Food Microbiology and Human Nutrition and Epidemiology at Wageningen University. She completed her studies with an internship at the Peninsula Technikon in Bellville, South Africa. In 2001 she was appointed by the division of Human Nutrition of Wageningen University, to conduct the PhD-project described in this thesis. This research was carried out at the Food Bioactives Group of RIKILT-Institute of Food Safety in Wageningen and the Centre for Nutrition and Health of the National Institute of Public Health and the Environment (RIVM) in Bilthoven. Since 2005 she works as a researcher on the prevention of overweight, at the Centre for Prevention and Health Services Research of the RIVM.

Publications

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Educational programme

Discipline specific courses

VLAG course 'Nutritional and lifestyle epidemiology', Wageningen, 2001
VARIAN course 'Principles and practice of modern HPLC', Bergen op Zoom, 2001
VLAG-CHN masterclass 'Nutrigenomics', Wageningen, 2001
RIVM-IMA course 'Epidemiologic data analysis', Bilthoven, 2002
RIKILT course 'LC-MS training' Micromass, Wageningen, 2002
NIHES course 'Conceptual foundation of epidemiologic study design', Rotterdam, 2003
NIHES course 'Introduction to data-analysis', Rotterdam, 2003
NIHES course 'Regression analysis', Rotterdam, 2003
NIHES course 'Survival analysis' Rotterdam, 2003
RIVM-IMA course 'Introduction to SAS', Bilthoven, 2004
VLAG masterclass 'Nutrition communication', Wageningen, 2005

Conferences

VLAG-CHN symposium 'Centre for Nutrigenomics', Wageningen, 2001
RIKILT symposium 'Bioactive compounds and health', Wageningen, 2001
VLAG-CHN thematic day 'Molecular epidemiology', Wageningen, 2002
RIVM conference '40-year results of the Seven Countries Study', Zutphen, 2002
VLAG-CHN thematic day 'Food safety', Wageningen, 2002
Pythochemical Society Europe conference 'Phytochemistry and biology of lignans', Walberberg, Germany, 2003
Federal Research Centre for Nutrition conference 'Phytoestrogens: benefits and risks for human health', Karlsruhe, Germany, 2003

Phytohealth conference 'Improving health through dietary phytoestrogens', Barcelona, Spain, 2004
Groupe Polyphénols conference 'XXII International conference of polyphenols', Helsinki, Finland, 2004
IEA-EEF conference 'European congress of epidemiology', Utrecht, 2006
NWO annual meeting 'Nutritional Science Community', Papendal, 2001-2005
ZonMw annual meeting 'Program Sensible Diet', Utrecht, 2002-2006

General courses

VLAG 'PhD week', Bilthoven 2001
CENTA course 'Scientific writing', Wageningen, 2002
NWO 'Talent day', Zeist, 2003
RIVM-BDA 'Introduction to Procite', Bilthoven, 2003
WGS course 'Career perspectives', Wageningen, 2005

Optional courses and activities

VLAG-Human Nutrition PhD study tour, 2001
Human Nutrition Meetings of Journal Club, 2001-2003
Human Nutrition Meetings of Brainstorm Club, 2001-2003
RIVM-CVG Meetings of Review Club 2003-2005
RIVM-CVG Lecture series, 2003-2005
RIVM-CVG Strategy day, 2003
Preparation of research proposal 2001-2005

Colofon

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