SPECIAL GUEST EDITOR SECTION

Flax Lignans—Analytical Methods and How They Influence Our Understanding of Biological Activity

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Flaxseed (*Linum usitatissimum* L.) is a major source of dietary intake of lignans by virtue of the high concentrations (0.7–1.5%) that are present in the seed. The principal lignan present in flaxseed is secoisolariciresinol diglucoside (SDG), which occurs as a component of a linear ester-linked complex in which the C6-OH of the glucose of SDG is esterified to the carboxylic acid of hydroxymethylglutaric acid. Also present in flaxseed and in resulting lignan extracts are significant quantities of 2 cinnamic acid glycosides. Our emerging understanding of the biological activity of flax lignans is based on studies using a variety of materials ranging from whole ground seed to pure SDG. The underlying assumption of most of these studies is that the biological activity of flax lignans results from their conversion to the mammalian lignans enterolactone (EL) and enterodiol (ED). There are, however, several intermediate compounds generated during the digestion and metabolism of flax lignans, including SDG and its aglycones and secoisolariciresinol (Seco), that are good candidates to be the principal bioactive molecule. This review will document the history of the development of lignan analytical methods and illustrate how analytical methods have influenced the interpretation of animal and human trials and our understanding of the biological activity of flax lignans.

When the history of the development of bioactive molecules from plant sources is examined, it is usually found that the development path follows a predictable pattern. Usually there is some association between the whole plant or part of the plant (i.e., the seed, flower, or root) and the biological activity of interest. This association usually has its origin in some form of traditional use that has developed over a long period of time and forms the basis of a research program to identify the biologically active component. Flax lignans, on the other hand, have a rather unusual discovery path. The discovery of their biological activity has its origin in a chance observation made in the late 1970s by researchers investigating changes in mammalian hormone levels (1, 2). Two unknown compounds were observed and subsequently identified as enterolactone [*trans*-2,3-*bis*(3-hydroxybenzyl-γ-butyrolactone (EL)] **1** (*see* Figures 1–4 for structures) and enterodiol [2,3-*bis*(3-hydroxybenyzl)butane-1,4-diol (ED)] **2** (3). Subsequently, the term mammalian lignans was coined and continues to be widely used to identify these compounds and their related derivatives. In 1982, the diet origin of these compounds was confirmed and the principal source identified as the lignan secoisolariciresinol [(+)-2,3-bis (methoxy-4-hydroxybenzl)butane-1,4-diol (Seco)] **3** (4). The most abundant source was determined to be flaxseed (*Linum usitatissimum* L.), in which the lignan occurs as secoisolariciresinol diglucoside (SDG) **4**. This compound had been isolated and identified in 1956 by Bakke and Klosterman (5), although no significance or biological activity was ascribed to the compound at that time. This review will document the parallel development of analytical methods and discovery of the biological activity of these compounds and show how the selective application of gas chromatography/mass spectrometry (GC/MS) analytical methodology has influenced our understanding of the biological activity of lignans in mammalian systems and how this has shaped our perception of what the biologically active molecule is. In this review, the focus will be on studies that examine the biological activity of isolated or semipurified compounds and analytical methods designed to detect these compounds. In most cases, studies with flaxseed or flaxseed meal precede the studies discussed in this review.

The Chronology

The history of flax lignans has 2 beginnings, the first in 1956 when Bakke and Klosterman (5) reported the isolation and purification of a diphenolic glycoside and gave it the common name SDG **4** (Table 1). The significance of the isolation procedure that was employed in 1956 was not appreciated until the early 1990s when researchers began to develop processes to isolate large quantities of SDG **4** for animal experimentation (37, 48); however, for 25 years SDG was just a scientific curiosity. Subsequent research confirmed that SDG did not exist in a free form in flaxseed but, rather,

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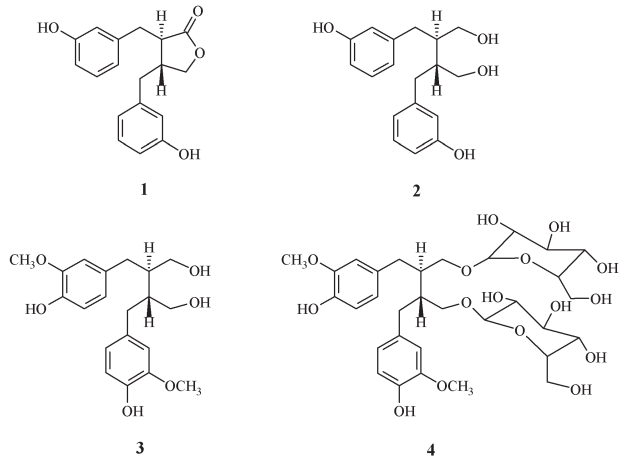

Figure 1. Structures of enterolactone (EL) 1, enterodiol (ED) 2, secoisolariciresinol (Seco) 3, and secoisolariciresinol diglucoside (SDG) 4.

was part of a soluble ester-linked complex **7** (Figure 3) that contained 3-hydroxyl-3-methyl-glutaric acid (HMGA) and a number of cinnamic acid glucosides **8** (R_1 or R_2 = O-glucose; Figure 4; 82, 83). The precise arrangement of the cinnamic acid glucosides remains to be determined; however, it is known that they are also linked to this complex by ester bonds through the carboxylic acid of the cinnamic acids.

The second beginning was in 1980 when 2 laboratories working independently on mammalian sex hormone analysis observed 2 unknown peaks in their GC traces (1, 2, 6; Table 1). Subsequently, these peaks were identified as EL **1** and ED **2** (3, 7, 8). Because these compounds contained a unique feature in that the sole aromatic hydroxyl group on each ring was in the *meta* position rather than the *para* position that is found in plant lignans, these compounds were referred to as mammalian lignans (11). Beginning about 2003, the term "enterolignan" appears in the literature as an alternative name to describe ED **2**, EL **1**, and related compounds (80).

Subsequently in 1981, it was shown that the colonic microflora were essential for the production of mammalian lignans (10, 11), and in 1982 it was shown that the mammalian lignans were originating in the diet (14), were undergoing enterohepatic circulation (Figure 5; 13), were excreted as conjugates (9), and that flaxseed was probably the richest single plant source of the precursors for these compounds (4).

The unfortunate legacy of this early research is the persistence of the GC-based analytical methodology long after it should have been displaced by high-performance liquid chromatography (HPLC) methods. Initially, no viable alternative existed because the concentrations of these compounds in urine and plasma were below the detection limits of early HPLC ultraviolet-visible (UV-Vis) detectors. The first generation of GC/MS instruments were now becoming more widely available, and LC/MS was not yet a viable technology. Therefore, for the next 15 years the analytical method of choice was GC/MS. In 1986, deuterated internal standards were introduced for GC/MS (22), and selected ion monitoring (SIM) was widely adopted as a means to increase the sensitivity of the technique, culminating in 1991 in the publication of an improved isotope dilution GC/SIM-MS method with internal standards for EL **1**, ED **2**, and matairesinol [dihydro-3,4-divanillyl-2(3*H*)-furanone (MAT)] **5** (25). This method was subsequently adapted for plant samples (43).

There were, however, 2 unintended consequences of this continued focus on GC-based analytical methodology. The first was the requirement for extensive sample preparation, including the necessity to make essentially water soluble polar compounds into nonpolar compounds that could be separated in a gas chromatograph. This usually involved a complicated purification protocol and the use of enzymes, and later the addition of acid hydrolysis (43) to remove sugars and sulfate groups followed by chemical derivatization prior to analysis.

The second unintended consequence was the use of SIM to increase the sensitivity. SIM can only be deployed when it is predetermined what compounds (and hence what ions) are being looked for in the sample. The consequence of these 2 technical issues was that the study of the biological activity of flax lignans was quickly focused on EL **1** and ED **2** to the exclusion of other possible biologically active molecules. This was in spite of reports of the presence of other lignans and metabolites in urine and plasma (18, 23).

During this period, many studies linked flax lignans to a range of biological activities (Table 1) principally related to hormone-dependent cancers, such as breast and colon cancer, but it was not until 1993 that the first animal study involving the feeding of the mammalian lignan precursor SDG **3** was published (28–30). Now it was possible to study the specific effects of feeding a purified compound to animals, to evaluate effects, and to begin to identify which compounds are biologically active. These initial studies with SDG were quickly followed by a series of experiments that significantly expanded the range of biological activities to include reduction in the progression of lupus nephritis (38), antioxidant activity (51), lowering of serum cholesterol levels (62), delayed progression of Type I (69) and Type II diabeties (71), reduction in melanoma metastasis (64), and reduction in plasma insulin-like growth factor I (70).

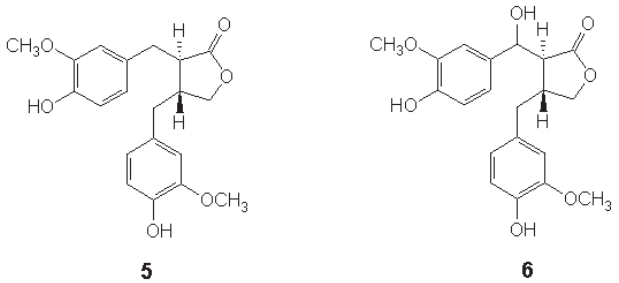

Figure 2. Structures of matairesinol (MAT) 5 and 7-hydroxymatairesinol (7-HMR) 6.

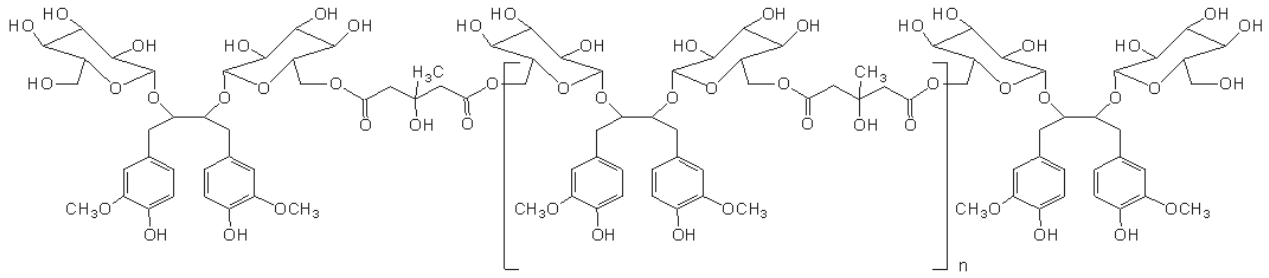

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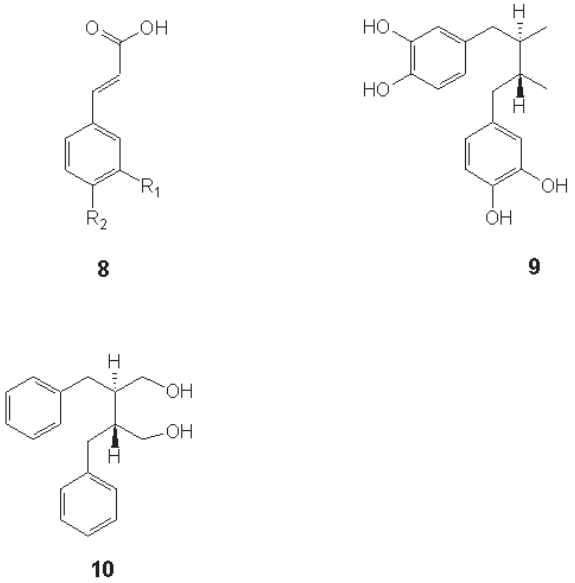
Figure 3. Proposed structure for the SDG-HMGA complex 7.

Biologically Active Molecules

The assumption that EL **1** (and possibly ED **2**) is the principal or most important biologically active molecule is not based on experimental comparative evidence but rather on the hypothesis that it is the active molecule. In the literature, there are numerous references to studies indicating that EL **1** has a range of biological activities; however, it is very rare for any of these studies to include other potential candidate molecules. For example, EL **1** was shown to stimulate the synthesis of sex hormone binding globulin (SHBG; 27), but only EL was tested in this study. EL and ED were shown to bind competitively with α-fetoprotein (84); however, the binding was weak relative to that demonstrated by nordihydroquaiaretic acid [2,3-bis(3,4-dihydroxybenzyl) butane (NDGA)] **9**, and the activity of mammalian lignan precursors, such as Seco **3** and SDG **4**, were not investigated.



8 **9** **10**

Figure 4. Structures of cinnamic acid glycosides 8, NDGA 9, and 2,3-dibenzyl-butane-1,4-diol (DBB) 10.

EL was shown to inhibit the proliferative effect of estradiol on MCF-7 breast cancer cells (85) and human colon tumor cells (49, 50). EL **1** and ED **2** were both shown to have inhibitory activity towards 5α-reductase, 17β-hydroxysteroid dehydrogenase (40), and cholesterol 7α-hydroxylase (86). In all of these studies, no other lignans were tested.

EL and 2 of its theoretical precursors were shown to have "moderate" aromatase inhibitory activity (36). In a later study, Seco was shown to have weak aromatase inhibitory activity (87). In a recent study, the aromatase inhibitory activities of EL **1**, ED **2**, Seco **3**, MAT **5**, and 7-hydroxymatairesinol (7-HMR) **6** were evaluated (88). Of the compounds tested, EL **1** was the most potent, with an IC_{50} (the concentration of drug required for 50% inhibition) of 8.9 μm; however, none of the compounds was tested at concentrations that adequately bracketed the expected IC_{50}, and, therefore, assessment of the relative inhibitory activity of these compounds was not possible.

Biosynthetic and Metabolic Pathways—Clues to Missed Opportunities

An underlying assumption that is often made in interpreting lignan data is that when plant lignan precursors are ingested, they pass to the large intestine and are completely metabolized to the mammalian lignans ED **2** and EL **1** prior to absorption into the body, or they are excreted. There are a number of examples of how the narrow focus on EL as the bioactive molecule may have distracted researchers from investigating other bioactive molecules.

As more researchers begin to look specifically for compounds like 7-HMR **6** and Seco **3** in plasma, these compounds are showing up in detectable quantities. In Lina et al. (89), 7-HMR plasma levels were approximately the same as the EL **1** levels in the low dose 7-HMR feeding group and were close to 6 times higher for the high 7-HMR dose. It is also usually assumed that metabolism stops at ED and EL. In 1989, 2,3-dibenzyl-butane-1,4-diol (DBB) **10** was reported to occur in

Table 1. Chronology of lignan analytical method development and discovery of the biological activity of flax lignans and related compounds

Biological activity	Chemistry and analysis
	1956–SDG **4** isolation by Bakke and Klosterman (5)
1980–Unknown GC peak observed in study of hormone profiles. Concentrations peaked in the luteal phase of the menstrual cycle (1, 2, 6)	1980–Unknowns identified as EL **1** and ED **2** (mammalian lignans) (3, 7, 8) and excreted as glucuronides (9). Lignan analytical methods based on classical steroid hormone analytical techniques
1981–Role of intestinal bacterial in the production of mammalian lignans demonstrated (10, 11)	1981–Mammalian lignans racemic (3, 7, 8)
1981–Excretion of mammalian lignans higher in young vegetarians (12)	1981–C18/ion exchange sample preparation GC and GC/MS analysis of trimethylsilyl (TMS) derivatives (10)
1982–Mammanlian lignans shown to undergo enterohepatic circulation (13; Figure 5)	
1982–EL **1** and ED **2** established as having diet origin (14)	
1982–Plant lignans identified as the source of mammalian lignans with SDG **4** from flaxseed the principal source (4). MAT **5** (*see* Figure 2 for structures) also a precursor	1982–Capillary GC method developed. Methods only check for EL **1** and ED **2** (15)
1982–Excretion of mammalian lignans lower in women with breast cancer than in the healthy population (15)	
	1983–Stable isotope GC-MS method (EL **1** and ED **2** only; 16)
1984–Adlercreutz (17) proposed that lignans protect against colon and breast cancer	1984–MAT **5** detected in urine (18)
1986–EL **1** inhibits Na+, K+-pump activity (19–21)	1986–Development of GC/SIM-MS method using deuterated external standards (EL **1** and EL **2**; 22)
	1989–Lariciresinol, isolariciresinol, and Seco **3** detected in urine by GC (23)
1991–Thompson et al. (24) reported the ability of human fecal microbiota to produce EL **1** and ED **2** from various food sources	1991–Improved isotope dilution GC/SIM-MS method with internal standards for EL **1**, ED **2**, and MAT **5** (25)
	1991–First HPLC method for analysis of SDG **4** in flaxseed described (26)
1992–EL **1** stimulates synthesis of SHBG by HepG2 liver cancer cells (27)	
1993–First studies with SDG **4** showing anticancer activity (28–30)	1993–HPLC analysis of plant lignans after acid hydrolysis (31)
1993–Antiestrogentic effect of SDG **4** demonstrated (32)	1993–HPLC analysis of plant lignans after hydrolysis with β-glucuronidase (33, 34)
	1993–GC ion mobility detector (EL **1** and ED **2**; 35)
1994–EL **1** inhibits aromatase activity (36)	1994–Base catalyzed hydrolysis of flax meal and extracts followed by reversed-phase (RP)-HPLC analysis (37)
1994–SDG **4** slows progression of symptoms of Lupus nephritis in animal models (38)	1994–SDG **4** present as both + and – isomers (39)
1995–EL **1** and ED **2** shown to inhibit 5α-reductase and 17β-hydroxysteroid dehydrogenase (40)	1995–Revised isotope dilution GC/SIM-MS method to include Seco **3** fraction that had previously been discarded (41)
1996–SDG **4** has protective effect on colon cancer (42)	1996–Revised isotope dilution GC/SIM-MS method adapted for plant samples (43)
1996–SDG **4** increases fecal β-glucuronidase activity (42, 44)	1996–HPLC analysis of SDG **4** content in flaxseed (45–47)
1996–SDG **4** feeding results in a dose dependent increase in urinary excretion of EL **1** and EL **2** (48)	
1996–EL **1** and EL **2** reduced proliferation of human colon tumor cells (49, 50)	
1997–Hydroxyradical scavenging activity of SDG **4** (51)	
1997–Seco **3** and Anhydroseco shown to have significant SHBG binding affinity (52, 53)	
1998–SDG **4** delays progression of MNU-induced mammary tumorigenesis (54, 55)	1998–Time-resolved fluoroimmunoassay for plasma EL **1** (56–58)

Table 1. ***(continued)***

1998–SDG **4** shown to lengthen estrous cycle in rats (59, 60)	1998–HPLC–coulometric array method for EL **1** and EL **2** in plasma, tissue, and urine (61)
1999–Reduction of serum cholesterol and hypercholesterolemic atherosclerosis by SDG **4** (62)	1999–Acid hydrolysis followed by GC analysis. Identification of isolariciresinol and pinoresinol in flaxseed (63).
1999–SDG **4** reduced experimental melanoma metastasis (64)	1999–Revised acid hydrolysis method followed by GC/MS analysis. Identification of enterofurn (65).
1999–Urinary excretion of EL **1**, ED **2**, and Seco **3** increase show dose dependent increase with flaxseed supplementation (66)	
1999–SDG **4** induced structural changes in the mammary gland that may potentially reduce mammary cancer risk (67, 68)	
2000–SDG **4** delays progression of Type I diabetes (69)	
2000–SDG **4** intake resulted in reduced plasma insulin-like growth factor I (70)	
2001–SDG **4** delays progression of Type II diabetes (71)	2001–HPLC–coulometric assay method for Seco **3**, ED **2**, EL **1**, MAT **5**, and other plant lignans (72)
2002–SDG **4** suppresses phosphoenol-pyruvate carboxykinase gene expression (73)	2002–HPLC method for the analysis of Anhydroseco generated by acid hydrolysis (74)
	2002–HPLC–Coulometric detection method for MAT **5** in plant samples (75)
	2002–LC/MS (ion trap) electrospray ionization (ESI) method for analysis of EL **1** and EL **2** in plasma and urine (76)
2003–SDG **4** inhibits chemically induced mammalian tumorigensis (77)	2003–LC/ESI-MS/MS (EL **1** and EL **2**; 78)
	2004–High-performance thin-layer chromatography method for SDG **4** (79)
	2004–LC/MS/MS method for lignans in food (Seco **3**, MAT **5**, lariciresinol, pinoresinol; 80)
	2004–HPLC–coulometric detection method expanded to include pinoresinol, medioresinol, syrinharesinol, isolariciresinol, Anhydroseco, and 7-HMR **6** (81)

mammalian urine (90) and was subsequently shown to affect aorta contraction and Ca^{2+} channels (91); however, since this early work, no subsequent studies have investigated the levels of this compound in plasma or urine. No definitive study exists to establish the dietary source of this compound in spite of the apparent significant biological activity reported for it and its close structural similarity to the mammalian lignan ED **2**.

In 2000, Wang et al. (92) identified 7 metabolites of SDG after anaerobic incubation with human fecal suspensions and isolated 2 different bacterial strains that appeared to be responsible for different steps in the metabolic pathway. This would suggest that several of these metabolites other than EL **1** and ED **2** could be absorbed rather than be further metabolized by gut microflora. In 1990, Hirano et al. (93) demonstrated that a number of mammalian lignans, including EL **1** and ED **2**, inhibited the growth of the human breast cancer cell line ZR-75-1. Of the 12 compounds tested, EL **1** and ED **2** were among the least effective in inhibiting cell growth, and DBB **10** was the most effective.

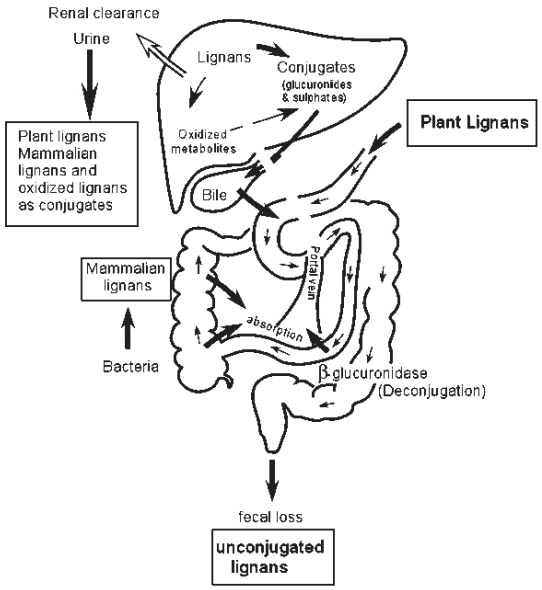


Figure 5. Enterohepatic circulation of lignans.

Table 2. Lignan levels in flaxseed

Analyte	SDG mg/g seed	Sample preparation	Analytical method
0.8 mg/g meal (as EL and ED)	≈1.0	Inclusion in diet, enzyme hydrolysis	GC/MS (4)
0.6 mg/g (as EL and ED)	1.4	Fecal fermentation	GC/MS (24)
	2.01	Enzyme hydrolysis	HPLC (34)
3.699 mg Seco/g	7.00	Enzyme + acid hydrolysis	GC/MS (43)
9.7–30.9 mg/g meal (SDG)	5.8–18.5	Base hydrolysis	HPLC (45–47)
0.7–1.4 mg Seco/g	1.37–2.74	Fecal fermentation	HPLC (94)
2 mg Seco/g	3.79	Not reported	HPLC (61, 95)
0.08–1.23 mg Seco/g	0.15–2.41	Enzyme	HPLC-electrochemical detection (96)
7.4–12.65 mg/g (as Seco and Anhydroseco)	14–24	Acid hydrolysis	GC/MS (65)
12.9–14.3 mg SDG/g flour	7.45–8.78	Base hydrolysis	HPLC (97)
	6.1–13.3	Base hydrolysis	HPLC (98)
Anhydroseco	6	Acid hydrolysis	HPLC (74)
	11.9–25.9	Base hydrolysis	HPLC (99)
45.8 mg SDG/g flour	≈27	Base hydrolysis	HPLC (100)

Analytical Methods for Mammalian Lignan Precursors

The analysis of lignan levels in flaxseed has also had a significant impact on the interpretation of animal feeding and diet intake studies. Only analytical methods in which quantitative data for lignan content of flaxseed are reported will be considered. Since the first report of SDG **4** levels in flaxseed in 1982, values have been reported that range from as little as 1 mg/g to as high as 25.9 mg/g (Table 2).

The concentrations of lignans reported in these studies tend to fall within the range of 1–4 mg SDG/g whole flaxseed when samples are subjected to enzyme hydrolysis or fecal fermentation. When acid or base hydrolysis is employed, the SDG **4** levels range from 4.2 to as high as 25.9 mg/g. This wide range of reported levels and the differences observed between enzymatic and chemical hydrolysis have significant implications for the interpretation of animal and human studies. Some of this variability is relatively easy to control because it reflects the variation due to cultivar and location (47, 94, 98); however, it does indicate the necessity to determine the lignan content of every lot of flaxseed used in animal or human experiments.

The second major source of variability is the method of hydrolysis used to release lignans from the ester-linked complex **7** (5, 37, 39, 83) that occurs naturally in flaxseed. Typically the rationale for using enzyme hydrolysis or fecal microflora fermentation is that this process is thought to more accurately reflect the release of soluble forms of the lignan present in the seed or, in the case of fecal microflora fermentation, to capture the contribution to mammalian lignan levels that may be coming from other precursors in the diet. There are several issues with these hypotheses. In the enzyme hydrolysis methods, the enzyme of choice (β-glucuronidase) is isolated from *Helix pomatia* (34). Although it is presumed that there are enzymes in the gastrointestinal (GI) tract, either of mammalian or bacterial origin, that are responsible for the hydrolysis of the lignan complex, none is available in the quantity needed for use in a routine assay. The fecal fermentation method does not address chemical or enzymatic modifications to the flax lignan complex that may occur in the stomach and small intestine and affect the bioavailablity of lignans. It has also been suggested that the activity of the fecal cultures is strongly influenced by the age, health, and diet of the human subjects that contribute the fecal material. Although no studies have yet been completed on the intestinal metabolism of flax lignans, a recent study by Glitsø et al. (101) of the metabolism of rye lignans in pigs indicated that unconjugated lignans were present in the ileum. The authors suggested that these plant lignans could enter the enterohepatic circulation (Figure 5) and potentially contribute to the observed biological activity. They also observed higher than expected levels of lignans in the feces when diets contained a high content of dietary fiber, suggesting that the analytical methods employed to determine the lignan content of the diet were underestimating the lignan content.

This wide range of values for the lignan content of flaxseed has significant implications for the interpretation of animal studies. As the availability of lignans in purified and semipurified forms increases, there is a natural progression occurring from studies that just focused on feeding flaxseed or flaxseed meal to those that compare the effects of flaxseed against a synthetic diet containing the equivalent amount of SDG to the whole flaxseed-containing diet. The challenge is

Table 3. Dietary intervention studies and epidemiological studies investigating the effects of diet on mammalian lignan levels

Study	Sample	Analytes
Diet effects (103)	Plasma	EL, ED
Multiethnic young women (104)	Urine	EL, ED
Chinese women and breast cancer (105)	Urine	EL, ED
Postmenopausal women (27)	Urine	EL, ED, MAT
Diet effects (106)	Urine	EL, ED, MAT
Premenopausal women (107, 108)	Feces, urine	ED, EL. MAT
Diet effects (109, 110)	Urine	EL, ED
Flaxseed in diet (95)	Urine	EL, ED, MAT
Diet effects (111)	Urine	EL
Post menopausal cancer risk (112)	Urine	EL
Dietary intake (113–117)	Plasma	EL
Breast cancer risk (118, 119)	Plasma	EL
Bone mineral density (120)	Urine	EL
Prostate risk (121)	Plasma	EL
Prostate and benign prostate hyperplasia (BPH; 122, 123)	Prostate tissues and plasma	EL, ED

to determine what would be the equivalent amount of SDG given the wide range of values reported (Table 2). Most of the early studies used SDG levels as determined by enzyme hydrolysis or fecal fermentation-based analytical methods (28, 29, 42, 44, 59, 60, 67) to determine what an equivalent SDG dose should be. All of these studies showed that SDG caused a similar biological effect to whole flaxseed; however, often the conclusion was that the SDG was not responsible for all of the activity seen in the whole flaxseed diet.

The conclusions of these studies suggesting that other components might be contributing to the biological activity need to be re-evaluated as a consequence of a recent review (102). In this review (102), it was reported that reanalysis of flaxseed used in a series of these animal studies, employing GC/MS analysis of base hydrolyzed extracts, determined that the SDG content was 13 mg/g compared to the value of 2 mg/g previously reported for this material based on enzyme hydrolysis and HPLC-UV analysis. It would now appear much more likely that the animals receiving a diet containing purified SDG received a lower dose than the animals on a supposedly equivalent SDG content in a whole flaxseed diet.

It may not be possible to determine the absolute SDG equivalency for whole flaxseed; however, it is increasingly clear that the SDG concentration in flaxseed as determined by enzyme hydrolysis or fecal fermentation-based analytical methods represents only the lowest concentration of soluble lignans available in the mammalian GI tract. When administering this level of isolated lignans does not appear to explain the biological activity observed in whole flaxseed, a high level of isolated lignans, closer to the concentrations determined by acid or base hydrolysis, should be evaluated. Only when administration of these higher concentrations still does not produce a similar response to feeding flaxseed or flaxmeal should other bioactives be considered.

Impact of Analytical Methods on the Design and Evaluation of Dietary Intervention and Epidemiological Studies

From the very earliest studies on lignans and human health, the focus has been on the mammalian lignans as the biologically active molecules, and this is dramatically illustrated in Table 3. There is no doubt that there are significant correlations between urinary excretion of mammalian lignans and lignan intake in the diet. There are also strong correlations between mammalian lignan levels and certain diseases, such as breast cancer; however, these correlations do not necessarily prove that the mammalian lignans are the main compounds responsible for the biological activity observed. Noticeably absent from these studies are any correlations with the plasma and urinary levels of Seco, and there was only sporadic inclusion of MAT in these studies. The immunological assay for EL is a relatively inexpensive tool for evaluating the lignan level in diets both in controlled feeding studies and in epidemiological studies, and it can be used very effectively to monitor compliance. Unfortunately, this technology is currently limited to detecting EL levels. The danger of the widespread use of immunological assays in diet intervention and epidemiological studies is that by focusing exclusively on EL, other potentially much more biologically significant interactions will be missed. For example, studies with 7-HMR **6** indicated that although this compound can be converted to EL **1**, it is also absorbed intact and excreted as

7-HMR conjugates (89, 124, 125). Analysis of blood drawn from subjects participating in clinical trials with the SDG lignan complex also indicated that Seco is present in significant quantities (*personal communication*, Flickinger, B.D., Archer Daniels Midland, Decatur, IL, 2006).

So why are so few animal and human studies being conducted in which the plant lignans, such as SDG **4**, Seco **3**, and MAT **5**, are being measured? In the early days it may have been due to the lack of standards, but right from the beginning EL **1** and ED **2** have been synthesized (126, 127), while SDG **4** and Seco **3** can be obtained in quantity by isolation from plant sources. The biological halflife of SDG **4** and Seco **3** appear to be quite short (unpublished data); however, relatively little information on the pharmacokinetics of any of these compounds is available. Seco **3**, 7-HMR **6**, and MAT **5** have very similar chromatographic properties compared to EL **1** and ED **2**. As some recent studies have shown, they can be quantified in plasma and urine (89), so there does not appear to be any technical reason why plant lignans are not routinely included in the analytical protocols for plasma and urine samples.

Conclusions

Analytical methods play a much larger role in influencing our understanding of biological systems than we are usually prepared to admit. Historically, the focus of analytical methods was on determining the mammalian lignans EL **1** and ED **2**. It was not until 1995 that it became apparent that other important metabolites, including Seco, were lost during the purification process (41). The focus on EL **1** and ED **2** as the biologically active molecules has also been shaped by a series of in vitro experiments in which only these compounds and none of the other lignans that we now know to be present in the plasma were tested. History may be correct and EL **1** and ED **2** may be the most important biologically active molecules, but to date there are few, if any, convincing experiments on which to base this assumption. Compounds such as Seco **3** and 7-HMR **6** are now much cheaper to isolate from plant sources, and these compounds need to be evaluated in much more detail.

The continuing widespread use of GC/MS as an analytical tool may still be necessary in a few limited situations where a highly sensitive assay is required; however, its use as a tool for the analysis of plant extracts should be discouraged. As HPLC/tandem mass spectrometry (HPLC/MS/MS) technology improves in sensitivity, it should become the preferred method for direct analysis of lignans and their conjugates.

As the supply of purified lignans such as the flax lignan SDG **4** and 7-HMR **6**, from Norway spruce (*Picea abies*; 128) becomes widely available, it will be possible to investigate the specific activities of these compounds and their metabolites. In these studies, it will be critical to analyze for all possible metabolites and to test all assumptions that are made as to what metabolites it would be expected to find.

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