

# **Sesame Seed Lignans**

**Diversity, Human Metabolism and Bioactivities**

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To my parents



## Abstract

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Sesame seed has been an important oilseed since ancient times. It contains 25% protein and 50% oil. Sesame seed possesses many health promoting effects, some of which have been attributed to a group of compounds called lignans (sesamin, sesamol, sesaminol and sesamolol). Sesame seed contains lignan aglycones in oil and lignan glucosides. Sesamin, the major sesame oil lignan, is known to reduce plasma cholesterol and to increase plasma  $\gamma$ -tocopherol in humans.

This thesis aimed at investigating the content of lignan and lignan glucosides in sesame seeds, human metabolism of sesamin and the effect of possible interaction between sesamin and phytosterols on the bioactivity of sesamin. To these purposes, two new lignan glucosides, sesaminol diglucoside and sesamolol diglucoside, were isolated from sesame seeds and characterized by LC-MS and NMR. A method for the analysis of sesame lignan glucosides was developed based on extraction of defatted sesame flour with 85% and 70% ethanol and using naringenin as internal standard and a reversed phase HPLC-DAD. A quantitative NMR technique was also developed to analyze the urinary metabolite of sesamin in humans based on using methylenedioxyphenyl signal ( $\delta$  5.91) in  $^1\text{H-NMR}$  spectra of deconjugated urine extracts to measure the metabolite(s) content.

A diversity of sesame seed oil and products were analyzed for their content of lignans and lignan glucosides. It was shown that sesame seeds contain almost 0.6% of lignans and 32% of lignans are in glucosylated form of sesaminol and sesamolol.

In a human study, volunteers ( $n=6$ ) consumed a single dose of muffins backed with sesame oil containing sesamin and sesamol. It was shown that almost 30% of sesamin ingested is excreted in urine in the form of a major catechol metabolite.

In an animal experiment, rats were fed diet containing two concentrations of sesamin and phytosterols. It was shown that phytosterols reduce the lipid modulating effects of sesamin on  $\gamma$ -tocopherol and dihomogamma-linolenic acid.

Overall, the findings from this thesis show that sesame seed is a rich source of lignans and that at least 30% of sesamin is absorbed in human, however the form of absorption is to be elucidated. Moreover, the biopotency of sesamin was reduced when the concentration of phytosterols was increased in the diet possibility because of reduction in the bioavailability of sesamin.

**Keywords:** Sesame seed, lignans, sesamin, sesamol, sesaminol, sesamolol, human metabolism, bioavailability, rats, cytochrome P450, CYP, tocopherol, fatty acid.

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## Appendix

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

### Papers I-VI

- I. Moazzami, A.A., Andersson, R.E. & Kamal-Eldin, A. 2006. HPLC analysis of sesaminol glucosides in sesame seeds. *Journal of Agricultural and Food Chemistry* 54: 633-638.
- II. Moazzami, A.A., Andersson, R.E. & Kamal-Eldin, A. 2006. Characterization and analysis of sesamol diglucoside in sesame seeds. *Bioscience, Biotechnology, and Biochemistry* 70: 1478-1481.
- III. Moazzami, A.A. & Kamal-Eldin, A. 2006. Sesame seed is a rich source of dietary lignans. *Journal of American Oil Chemist Society* 83: 719-723.
- IV. Moazzami, A.A., Haese, S., Kamal-Eldin, A. 2006. Lignan contents in sesame seeds and sesame seed products. Manuscript.
- V. Moazzami, A.A., Andersson, R.E. & Kamal-Eldin, A. 2006. Human metabolism and urinary excretion of sesamin. Submitted
- VI. Moazzami, A.A., Frank, J., Lundh, T., Vessby, B & Kamal-Eldin, A. 2006. Phytosterols inhibit lipid modulating effects of sesamin in rats. Manuscript.

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## List of abbreviations

CAR	constitutive androstane receptor
CHD	coronary heart disease
DSF	defatted sesame flour
ER	estrogen receptor
HDL-C	high density lipoprotein cholesterol
HepG2	human hepatoma cell line G2
HPLC-DAD	high performance liquid chromatography diode array detector
IL-6	interleukin 6
LC-MS	liquid chromatography mass spectroscopy
LDL-C	low density lipoprotein cholesterol
LTB <sub>4</sub>	leukotriene B <sub>4</sub>
LXR	liver X receptor
NMR	nuclear magnetic resonance spectroscopy
PAL	phenylalanine ammonia lysase
PGD <sub>2</sub>	prostaglandin D <sub>2</sub>
PGE <sub>2</sub>	prostaglandin E <sub>2</sub>
PGF <sub>2</sub> $\alpha$	prostaglandin F <sub>2</sub> $\alpha$
PGH <sub>2</sub>	prostaglandin H <sub>2</sub>
PGI <sub>2</sub>	prostaglandin I <sub>2</sub>
PGG <sub>2</sub>	prostaglandin G <sub>2</sub>
PXR	pregnane X receptor
RP	reversed phase
S <sub>2</sub> G	sesaminol diglucoside
S <sub>3</sub> G	sesaminol triglucoside
TC	total cholesterol
TLC	thin layer chromatography
TNF- $\alpha$	tumor necrosis factor- $\alpha$
TxA <sub>2</sub>	thromboxane A <sub>2</sub>



## Sesame

Sesame belongs to the genus *Sesamum*, one of 16 genera in the family pedaliaceae. The genus *Sesamum* comprises about 35 wild species besides the only cultivated species, *Sesamum indicum*. The name sesame comes from the Arabic word “simsim”. Linnaeus and Brdigian & Harlan (1986) cited India as the origin of sesame, however there is also belief that the actual origin was Africa, where many wild species are found (Namiki, 1995). In the tale of Ali Baba and the 40 thieves, in the 'Thousand and One Nights' the password 'open sesame' may have been chosen because the ripe seeds burst from their pods suddenly with a sharp 'pop' like the springing open of a lock.

Sesame is a broad-leaf summer crop with bell-shaped flowers and opposite leaves. It is an annual plant that can reach 1-1.8 m high. The plant prefers fertile, well-drained, and neutral to slightly alkaline pH. Sesame is cultivated in tropical areas and needs growing season of 110-150 frost free days. The best air temperature is 30-35°C and the soil temperature should not be lower than 20°C. Flowering starts about 40 days after planting the seeds and continues for almost another 40 days. The flowers then develop into seed pods (capsules), which contain 70-100 seeds each. The color of seeds varies from white to brown, gold, gray, violet and black. Seeds are tiny and weigh 2-3.5 g/1000 seeds. Seed yield ranges from 350 to 1700 kg/ha depending on the varieties used and cultivation techniques. According to FAO, the average yield is almost 500 kg/ha.

*Sesamum indicum* is the major commercial source of sesame seeds and primarily grown in India, China, Mexico and Sudan. The production of sesame seed in 2002 was reported as 2.9 million tons world-wide, which puts sesame as the 8<sup>th</sup> oil crop (FAOSTAT, www.fao.org). The production of sesame seeds is low in comparison with soybean with 180 million ton production. This is mainly because of the requirement of manual harvest procedures since the sesame plant should be harvested before the seed capsules get open on the field and the seeds are lost. Following the discovery of a non-dehiscent (non-shattering) mutant by Langham in 1943, plant-breeding efforts began toward developing high-yield shatter-resistant varieties. Sesame plant with semi-shattering and non-dehiscent, in which the capsules hold the seeds when they get dry, provides advantages of mechanized harvest over traditional shattering plants.

Sesame seeds contain 25% protein and 50% oil. Sesame protein is slightly low in lysine but rich in other amino acids specially methionine, cystine, arginine and leucine. Sesame oil contains linoleic acid (37-47%), oleic acid (35-43%), palmitic (9-11%) and stearic acid (5-10%) with trace amount of linolenic acid (Kamal-Eldin *et al.*, 1992a). Sesame seeds contain a group of compounds, called lignans, to which many health promoting effects are attributed.

Sesame seeds are used in the preparation of a number of food products. In the Far East, sesame seeds are roasted (180-200°C) and their oil is extracted and sold as roasted sesame oil in the market. Crude and refined sesame oils are also available

in other parts of the world. Sesame oil is used as a salad or cooking oil, in shortenings, margarine and to marinate meat and vegetables. Sesame oil was the preferred cooking oil in India till the introduction of groundnut (peanut) oil. Refined sesame oil is mainly used in pharmaceutical and cosmetic products. Sesame seeds can be made into a paste called tahini and a Middle Eastern and Indian confection called halva. In India, parts of the Middle East and Far East Asia, popular products are made from sesame mixed with honey or syrup. Sesame flavor is also very popular in Korean cuisine. The leaves of the sesame plant are also used in Korean cuisine as a type of wrap, eaten with meat and other vegetables (a traditional dish). Sesame seeds are also sprinkled onto some sushi style foods. East Asian cuisines, like Chinese cuisine use sesame seeds and oil in some dishes. Japanese cuisine uses sesame seeds in many ways e.g. to make goma-tofu, which is made from sesame paste and starch. Whole seeds are found in many salads and baked snacks as well. In tempura restaurants, mixtures of sesame and cottonseed oils are used for deep-frying.

### **Sesame seed lignans: chemistry and biosynthesis**

In 1927, Robinson recognized that a common feature of many natural products is a  $C_6C_3$  unit (i.e. a propylbenzene or phenylpropanoid skeleton). In a review of natural resins, Haworth (1936) proposed that the class of compounds derived from two  $C_6C_3$  units possessing  $\beta,\beta'$ -linkage (8-8' bond) should be called lignans (his original spelling was lignane but the 'e' was deleted in subsequent publications). Sesame seeds contain a diversity of furofuran lignans, which can be divided into two types:

#### *Oil-soluble (or oil-dispersed) lignans (Figure 1)*

Sesamin (1) and sesamol (2) have been considered the major oil-soluble lignans of sesame seeds (Namiki *et al.*, 1995). However, small amounts of sesaminol (3), Piperitol (4), sesamolol (5), pinoselinol (6), (+)-episesaminone (7), hydroxymatairesinol (10), allohydroxymatairesinol (11) and larisiresinol (12), which possess free phenolic groups and therefore, antioxidant activity have been reported in sesame seeds (Fukuda *et al.*, 1985, 1988a; Osawa *et al.*, 1985; Marchand *et al.* 1997; Nagashima & Fukuda, 2004). Moreover, the presence of other lignans in the wild species of genus *Sesamum* like sesangolin (8) in *Sesamum angolense* and *Sesamum angustifolium* and 2-episesalatin (9) in *Sesamum alatum* has been reported (Jones *et al.*, 1962; Kamal-Eldin & Appelqvist, 1994; Kamal-Eldin & Yousif, 1992b). The range and some physical and chemical properties of sesame seed lignans are addressed in **Table 1**.

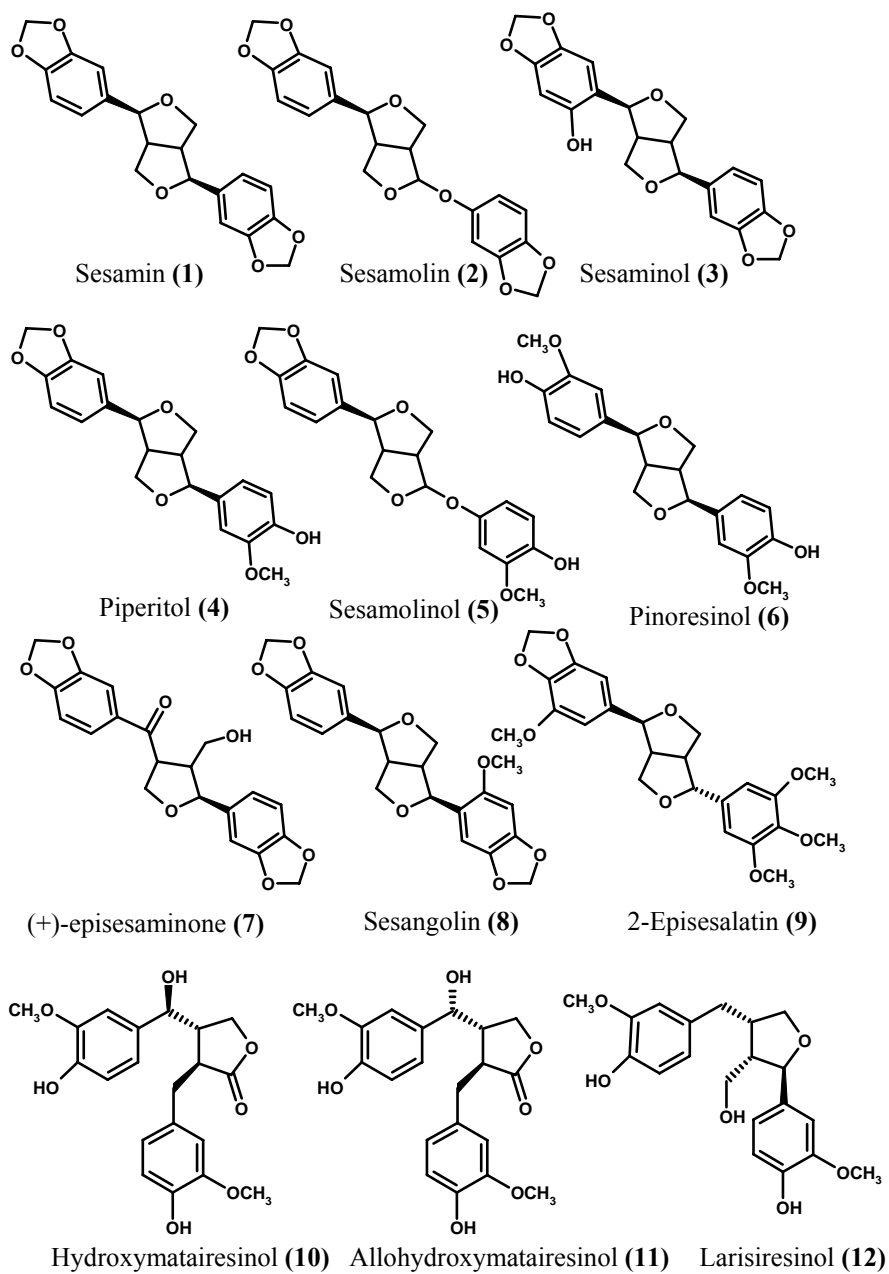


Figure 1. Sesame oil-soluble or oil dispersed lignans. The numbering is used from now on to refer to the corresponding lignan when the structure is not provided.

### Glucosylated lignans (Figure 2)

These are present in defatted sesame flour (DSF). Because antioxidant activity was developed after treatment of DSF with  $\beta$ -glucosidase, the presence of water-soluble and potentially antioxidant lignans in sesame seeds was investigated. Sesaminol triglucoside (13), sesaminol diglucoside (14a), sesaminol monoglucoside (15), pinoresinol triglucoside (16), two isomers of pinoresinol diglucoside (17a,b), pinoresinol monoglucoside (18), were then isolated and characterized (Katsuzaki *et al.*, 1992, 1993, 1994a,b).

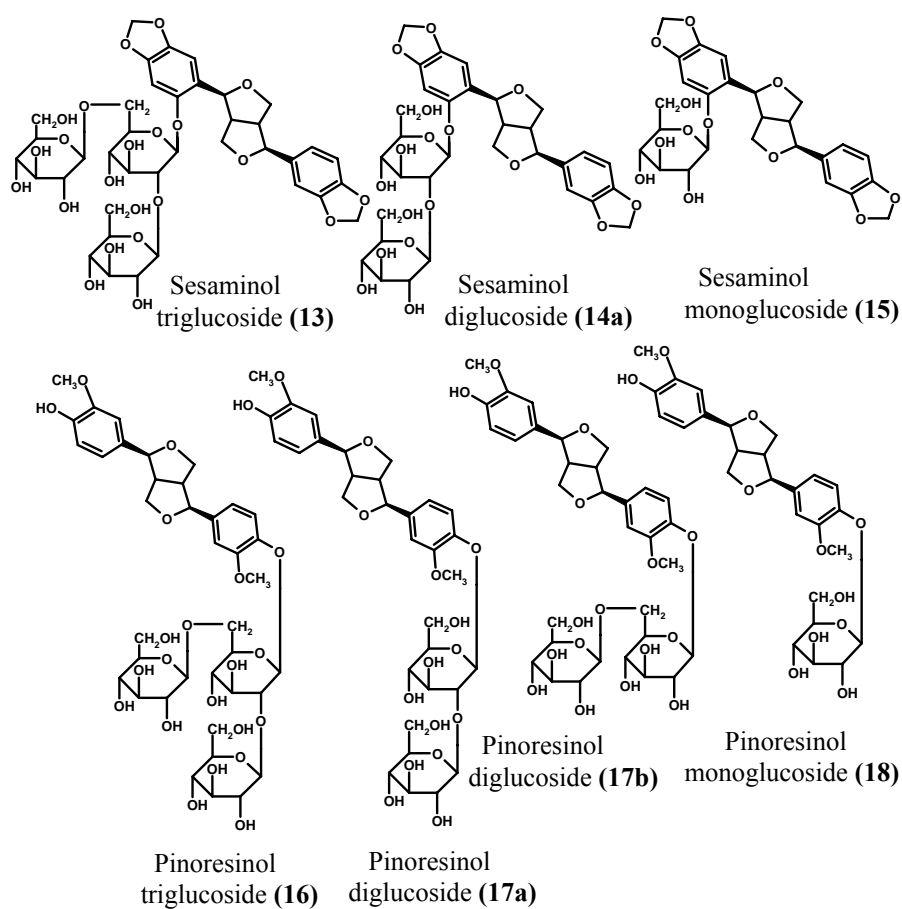


Figure 2. Lignan glucosides in defatted sesame flour isolated by Katsuzaki *et al.* (1992, 1993, 1994a,b). The numbering is used from now on to refer to the corresponding lignan glucoside when the structure is not provided.

Table 1. Range, molecular formula, UV absorption ( $\lambda_{\max}$ , nm) and extinction coefficient ( $\epsilon_{\max}$ , for 1 cm cell) of major sesame seed lignans and lignan glucosides.

	Range mg/100 seed	Molecular formula Mwt	$\lambda_{\max}$ (nm)	$\epsilon_{\max}$ (g <sup>-1</sup> L)
Sesamin <sup>a</sup>	77-930	C <sub>20</sub> H <sub>18</sub> O <sub>6</sub> 354	287	23.02 <sup>d</sup>
			236	26.01
Sesamolin <sup>a</sup>	61-530	C <sub>20</sub> H <sub>18</sub> O <sub>7</sub> 370	288	21.79 <sup>d</sup>
			235	24.85
Sesaminol <sup>b</sup>	0.3-1.4	C <sub>20</sub> H <sub>18</sub> O <sub>7</sub> 370	238	3.99 <sup>e</sup>
			295	3.85
Sesamolinal <sup>c</sup>	6-28	C <sub>20</sub> H <sub>20</sub> O <sub>7</sub> 372	231	3.95 <sup>e</sup>
			287	3.8
Sesaminol Triglucoside <sup>c</sup>	14-91	C <sub>38</sub> H <sub>52</sub> O <sub>24</sub> 856	235	3.97 <sup>f</sup>
			288	3.88
Sesaminol Diglucoside <sup>c</sup>	8.2-18.3	C <sub>32</sub> H <sub>40</sub> O <sub>18</sub> 694	236	4.0 <sup>f</sup>
			290	3.94
Sesaminol monoglucoside <sup>c</sup>	5.4-19.5	C <sub>26</sub> H <sub>28</sub> O <sub>12</sub> 532	236	3.80 <sup>g</sup>
			290	3.82

<sup>a</sup> The range was based on the data reported previously by Fukuda et al., 1988a; Beroza & Kinman, 1955 and Hemalatha & Ghafoorunissa, 2004.

<sup>b</sup> The range was based on the data reported previously by Fukuda et al., 1988a.

<sup>c</sup> The range was based on the data reported previously by Ryu et al., 1998.

<sup>d</sup> Measured in isoctane (Budowski & Markley, 1951).

<sup>e</sup> Measured in chloroform (Osawa et al., 1985; Nagata et al., 1987).

<sup>f</sup> Measured in water (Katsuzaki *et al.*, 1994b).

<sup>g</sup> Measured in methanol (Katsuzaki *et al.*, 1994b).

Sesamolin (2) and sesamolinal (5) possess an oxygen bridge between their benzene and furofuran rings (Namiki, 1995). This feature has not been observed in the lignan structure of any other genus than *Sesamum*. However, sesamin (1) has been identified in several species of plants (Parmar *et al.*, 1997). It was also identified in stem and root bark of some tree species (Chen *et al.*, 1999; Ju *et al.*, 2001; Meselhy 2003 and Wang *et al.*, 2003). Some of the sesame seed lignans including sesamin, sesamolin, sesamolinal, sesaminol, sesangolin, 2-episesalatin, and (+)-episesaminone possess methylenedioxyphenyl group (-O-CH<sub>2</sub>-O-) in their molecular structures, a group that has also been observed in the structure of other lignans like dibenzocyclooctadiene lignans (Chang *et al.*, 2005). The protons H-8 and H-8', on the bridgehead carbons have *cis* stereochemical configuration in sesame seed lignans, which can possibly be explained by stereoselective bimolecular phenoxyl radical coupling in the presence of a dirigent protein during the biosynthesis of lignans (Davin *et al.*, 1997).

Phenylalanine and tyrosine serve as precursors to an enormous number of plant substances including tannins, polymeric lignin and lignans. Phenylalanine can first be converted to cinnamic acid by the action of the enzyme phenylalanine ammonia lyase (PAL). A series of enzymatic hydroxylation, methylation and reduction leads to coumaric acid, caffeic acid, ferulic acid and eventually E-coniferyl alcohol (**Figure 3**) (Mathews *et al.*, 2000). However, it was claimed that [1-<sup>14</sup>C] labeled tyrosine was incorporated into sesamin (1) when administered to the cell suspension cultures of *Sesamum indicum* (Jain & Khanna., 1973). It was also demonstrated that E-coniferyl alcohol undergoes stereoselective coupling to afford (+)-pinoresinol in *Sesamum indicum* seeds. (+)-Pinoresinol is then metabolized further in maturing seeds to afford (+)-piperitol, and (+)-sesamin by introduction of methylenedioxyphenyl groups (Kato *et al.*, 1998). One-electron oxidation of the monolignol, E-coniferyl alcohol, results in random bimolecular radical coupling and the production of a mixture containing (±)-dehydroconiferyl alcohols, (±)-pinoresinol and (±)-guaiacylglycerol 8-O-4'-coniferyl alcohol. However, it was shown that in the presence of a 78-kD dirigent protein a stereoselective bimolecular coupling results in the production of (+)-pinoresinol (Davin *et al.*, 1997) and that just (+)-pinoresinol can be further metabolized to (+)-piperitol, (+)-sesamin and (+)-sesamol (Kato *et al.*, 1998) (**Figure 3**). Lignans formation is developmentally regulated and depends on the stage of seed maturity (Kato *et al.*, 1998). The most mature seeds at eight weeks efficiently convert (+)-pinoresinol into (+)-piperitol and (+)-sesamin, while younger seeds have higher conversion to (+)-sesamol (Jiao *et al.*, 1998). Later, it was shown that the content of sesamol (3) is increased at the end of seed maturation (Ono *et al.*, 2006) (**Figure 3**). It was suggested that microsomal preparation of sesame seeds catalyzes methylenedioxyphenyl formation *via* an O<sub>2</sub>/NADPH/chytochrome P-450 dependent transformation of (+)-pinoresinol into (+)-piperitol (Jiao *et al.*, 1998). Possible subsequent metabolic steps of the conversion of (+)-pinoresinol into (+)-piperitol and (+)-sesamin were suggested by Jiao *et al.* (1998). Recently, it was shown that the synthesis of sesamin from pinoresinol is catalyzed by CYP81Q1 in two steps (**Figure 3**) (Ono *et al.*, 2006). However, the mechanism of oxygen insertion between furfuran and benzene rings to form sesamol and the pathway to sesaminol are yet to be elucidated.

Sesamin (1) has been suggested to undergo further metabolism to form (+)-episesaminone (7), a keto-lignan, which was isolated from commercially available unroasted and unbleached sesame seeds as well as freshly harvested seed tissue (Marchand *et al.* 1997). Given that (+)-episesaminone can exist in its lactol form under basic condition, only a single oxidation step from (+)-sesamin to (+)-episesaminone is required (Marchand *et al.* 1997).

Changes in metabolism occur during germination of sesame seeds (Kuriyama *et al.*, 1995 and Ishiyama *et al.*, 2006). It was shown that in the early stage of germination, up to 48 h, sesamin (1) and sesamol (2) almost disappear and the content of sesaminol diglucoside (14b) increases. (+)-Episesaminone tri- and diglucosides are also generated during early stages of germination. These lignan glucosides existed in germinated sesame seeds for at least seven days after their appearance (Kuriyama *et al.*, 1995).



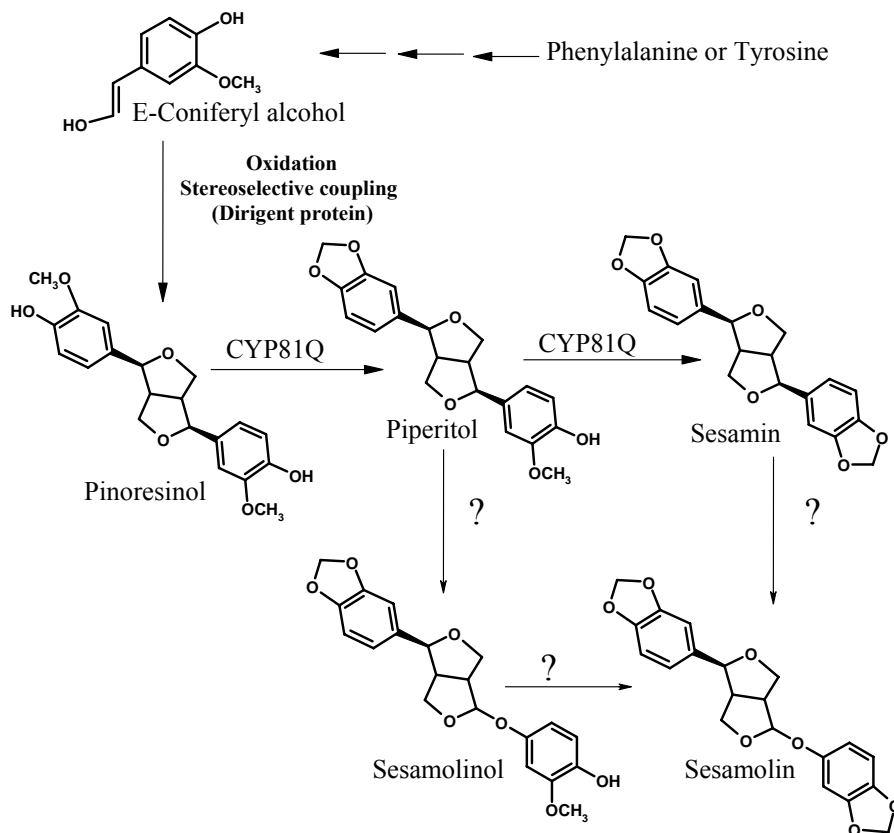


Figure 3. Biosynthesis of lignans from phenylalanine or tyrosine. Stereoselective coupling of two E-coniferyl alcohol is performed in the presence of a dirigent protein. Pinoresinol can be further metabolized to sesamin and sesamolol. The pathway to sesamolol, sesamololol and sesaminol are yet to be elucidated.

### Lignan transformation during refining and antioxidative activity

Roasted sesame oil has been considered as highly resistant to oxidative deterioration. It was shown that the rate of absorption of oxygen by sesame oil was lower than that for other edible oils and it was more stable under prolonged storage regarding both rancidity and free fatty acid content in comparison with other oils used in therapeutics (Budowski & Markley, 1951).

Three different types of sesame oil, including crude (virgin), roasted and refined sesame oils are produced. Sesame oil from unroasted sesame seeds is extracted with an expeller or solvent. This oil can undergo alkaline refining, bleaching with acidic clay and deodorization, as done for other vegetable oils, to produce refined sesame oil. On the other hand, roasted sesame oil, extracted from roasted sesame

seeds, is only filtered to remove contaminants. Comparison of autoxidation of commercial oils at 60°C revealed that soybean, rapeseed, safflower and corn oil began to oxidize after 5-20 days incubation, while refined sesame oil was oxidized after 35 days. Under this condition, roasted sesame oil was shown to remain unaltered even after 50 days (Fukuda *et al.*, 1988b).

Refined sesame oil was reported to contain some amount of sesaminol (6-60 mg/100g oil) (Fukuda *et al.*, 1986a). During the bleaching, in the presence of acidic clay and heat, sesamol is first decomposed into sesamol and oxonium ion by protonolysis and then a new carbon-carbon bond is formed to produce sesaminol. Therefore, an intermolecular transformation was suggested for conversion of sesamol to sesaminol during bleaching (Fukuda *et al.*, 1986b). Epimerization of the sesame oil lignans also happens during bleaching, in which episesamin and episesaminol are formed (Fukuda *et al.*, 1986a) (**Figure 4**).

Even though, the content of  $\gamma$ -tocopherol is reduced during refining of sesame oil, the refined oil has a stronger antioxidant activity in comparison with the crude oil. The antioxidant activity of sesaminol is roughly equal to those of sesamol and  $\gamma$ -tocopherol by the thiocyanate method. Moreover, sesamol that is also produced from sesamol during bleaching is nearly removed by subsequent deodorization. Therefore, the antioxidant activity of refined sesame oil was mainly attributed to sesaminol and residual  $\gamma$ -tocopherol (Fukuda *et al.*, 1986a).

The quality (flavor, color and oxidative stability) of the roasted sesame oil depends mainly on the roasting conditions. The degrees of browning and oxidative stability of the roasted sesame oil are increased by higher roasting temperature. The content of sesamol is not correlated with the increase in roasting temperature, even though it was shown that sesamol is also produced from sesamol during roasting by thermal decomposition (Fukuda *et al.*, 1986b). It was shown that sesamol, sesamol, sesamin,  $\gamma$ -tocopherol, browning materials can not individually account for the high oxidative stability of roasted sesame oil, and a system composed of all these sesame oil components synergistically protect roasted sesame oil against oxidative deterioration (Namiki 1995).

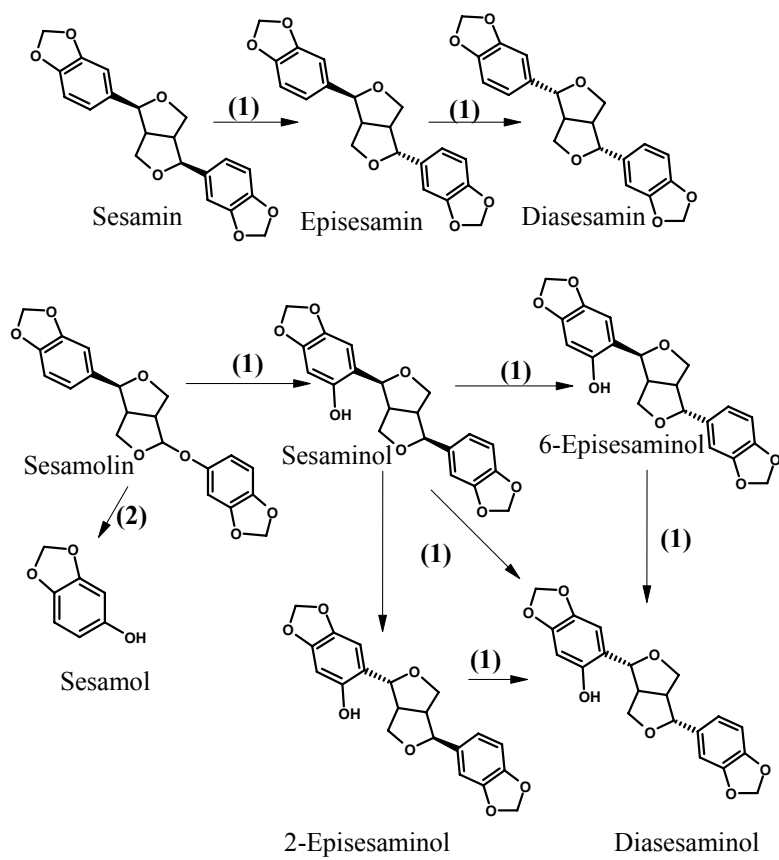


Figure 4. Transformation of sesame oil lignans during bleaching with acidic clay. (1) Bleaching by acidic clay. (2) Hydrolysis or heating.

## **Bioavailability and metabolism of sesame seed lignans**

The term “bioavailability” was initially introduced in the field of pharmacology and pharmacokinetics, where it was defined as “the fraction of a given dose that reaches the systematic circulation unchanged (Rowland & Tozer, 1995). Bioavailability is commonly applied to both rate and extent of drug or dietary compounds input into the systematic circulation. Absorption is defined as the process by which unchanged drug proceeds from the site of administration to the site of measurement within the body, which is the general circulation. For an orally administered compound, there are several possible sites of loss before it reaches the general circulation. One site is gastrointestinal lumen where decomposition may occur. However, if the drug or dietary compound survives the destruction in the lumen, then it can be metabolized by enzymes when it passes through the membranes of the gastrointestinal tract. Taking one step further, drugs or dietary compounds, which are not absorbed through the lymph, pass into the portal vein where they are carried to the liver and undergo further metabolism. Indeed, loss at any site prior to the site of measurement contributes to a decrease in systematic absorption and bioavailability. The gastrointestinal tissues and the liver are often sites of elimination and the requirement for dietary compound to pass through these tissues, prior to reaching the systematic circulation causes the extent of absorption to be dependent on elimination. The loss of a dietary compound for the first time during absorption, through sites of elimination such as the gastrointestinal membranes and the liver, is known as the first-pass effect (Rowland & Tozer, 1995).

In food products, bioaccessibility is defined as the amount of an ingested food compound that becomes available for absorption in the gastrointestinal tract. In this context, the bioavailability is also dependent on the bioaccessibility of the compound. In general, bioaccessibility is an integration of food matrix and all luminal events. In order for food components to be absorbed, they must be released from the food matrix and be presented to the brush-border of small intestine. This depends on the state of food (raw or processed), particle size, digestive enzymes, composition of meal and the presence of bile acids and salts. For example, it was shown that the bioavailability of enterolignans in human is enhanced by milling and crushing of flaxseed (Kuijsten *et al.*, 2005).

Most dietary phenolic compounds are in O-glycoside form and their hydroxyl groups, which are relevant for antioxidant activity, are blocked. Glucosidases in tissues or from bacteria in the gut remove the conjugated carbohydrate and the phenolic compounds are active antioxidants again (Stahl *et al.*, 2002). Xenobiotic metabolizing enzymes, such as cytochrome P450 (CYP450), conjugating enzyme of phase II biotransformation (e.g. UDP-glucuronosyl transferases or phenol sulphotransferases) play a major role in the metabolism of dietary phenolic compounds including antioxidants and lignans. Metabolic processes may considerably change the function of a particular compound, and metabolism generally results in the formation of derivatives that are more suitable for excretion than the parent molecules. Moreover, compounds generated by the transformations may be more or less bioactive than the parent compounds. Nakano

*et al.* (2006) suggested that sesamin metabolites generated after incubation with rat liver microsomes (Nakai *et al.*, 2003) induce an endothelial nitric oxide-dependent vasorelaxation.

Knowledge about the metabolism, and identification of metabolites is thus vital to the understanding of the mechanism of bioactivity and ultimately the identification of active compounds. It is also important for assessing the risk that might be associated with toxic effects of active dietary compounds (Stahl *et al.*, 2002).

A small amount of sesamin (0.1%) was shown to be absorbed through the lymph in rats (Hirose *et al.*, 1991; Umeda-Sawada *et al.*, 1999) and sesamin was also detected in the plasma of human volunteers after a single dose of sesame seeds (Penalvo *et al.*, 2005). The metabolism of sesamin has also been investigated *in vitro* and *in vivo* (**Figure 5**). Incubation of sesamin with liver microsomes causes the cleavage of methylenedioxyphenyl group in the molecule to form catechol derivatives (Nakai *et al.*, 2003). Moreover, it was shown that sesamin can undergoes further cleavages in its furofuran ring after 24 hour fermentation with human fecal microflora *in vitro* (Penalvo *et al.*, 2005) or administration to rats (Liu *et al.*, 2006).

Mammalian lignans, enterolactone and enterodiol (**Figure 5**) are the end-products of dietary lignan fermentation by mammalian intestinal microflora (Borriello *et al.*, 1985; Heinonen *et al.*, 2001 and Saarinen *et al.*, 2000). It was shown that the consumption of a single dose of sesame seeds by four volunteers increased the plasma concentration of enterolactone and enterodiol (Penalvo *et al.*, 2005). Coulman *et al.* (2005) also showed that the urinary excretion of mammalian lignans is increased after four week intervention with sesame bars containing 25 g seed. Little is known about the contribution of different sesame seed lignans in the production of mammalian lignans. However, it was shown that sesamin, the major sesame oil lignan, is converted to enterolactone, enterodiol and three other intermediate metabolites after 24 hour fermentation with human microflora *in vitro* (Penalvo *et al.*, 2005).

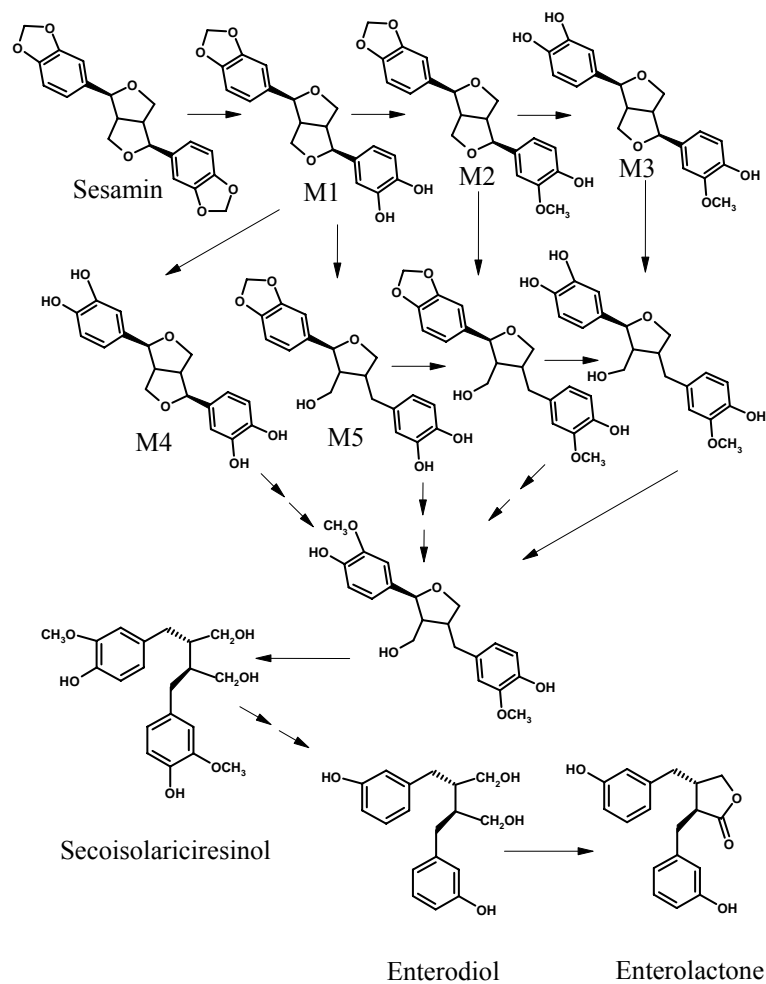


Figure 5. Possible pathway of sesamin metabolism in rats as suggested by Liu *et al.*, 2006. M1-4 were produced after incubation of sesamin with rat liver microsomes. M1, M4 and M5 were produced after 24 hour fermentation with human fecal microflora *in vitro* (Penalvo *et al.*, 2005)

## **Bioactivity of sesame seed and its lignans**

Sesame seed has been an important oil-seed since ancient times (Namiki, 1995). It was claimed in ancient Chinese books that consumption of sesame seeds (*Chih-Ma* in Chinese) provides increased energy and prevents aging. Sesame oil (*Tila* in ancient Indian language, Sanskrit) has been used as a domestic Ayurvedic medicine (roughly translated to science of life). Recently, scientific studies have attributed many of health-promoting effects of sesame seed to its lignans specially sesamin, which is the major oil-soluble lignan.

### *Reduction of cholesterol*

Coronary heart disease (CHD) is the number one cause of morbidity and mortality in industrialized western countries. A positive relationship between high blood cholesterol concentrations, particularly low-density lipoproteins cholesterol (LDL-C), and the incidence of atherosclerosis and CHD has been clearly established. Lowering blood cholesterol by dietary intervention is one of the first measures in prevention of CHD (Assmann *et al.*, 1999). Sesame oil and lignans have been investigated for their cholesterol-lowering effect. It was observed that sesame oil tended to reduce serum cholesterol levels in rats compared to corn oil in spite of the comparable fatty acid composition of the two oils (Koh, 1987). Hirose *et al.* (1991) showed that serum and liver cholesterol were reduced in rats fed diet containing 0.5% sesamin. They demonstrated that the hypocholesterolemic activity of sesamin can, at least in part, be explained by the inhibition of the intestinal absorption of cholesterol as reflected by the significant reduction in cholesterol in the thoracic lymph and a significant reduction in the activity of liver microsomal 3-hydroxy-3-methylglutaryl coenzyme A reductase, which is a rate limiting enzyme in biosynthesis of cholesterol. Consumption of 32 mg sesamin capsules for 4 weeks followed by 65 mg sesamin capsules for 4 weeks reduced total cholesterol (TC) (9%), LDL-C (16.5%) and apoprotein B (10.5%) in 12 males with hypercholesterolemia (Hirata *et al.*, 1996). In another study, 21 hypercholesterolemic subjects consumed 40 g of roasted sesame seeds for 4 weeks. The results showed that the sesame diet significantly decreased the levels of serum TC (6.4%) and LDL-C (9.5%). However, the effect of sesame seeds on cholesterol disappeared when patients stopped the consumption of sesame diet (Chen *et al.*, 2005). Wu *et al.* (2006) also observed similar reductions in TC and LDL-C in 24 postmenopausal subjects following a 5-week intervention with 50 g pulverized roasted sesame seed. High density lipoprotein cholesterol (HDL-C) was unchanged in all mentioned human studies after intervention of sesamin or sesame seed (Hirata *et al.*, 1996; Chen *et al.*, 2005 and Wu *et al.*, 2006). Since the diet can be an effective mean to lower blood levels of total and LDL cholesterol (Delahanty *et al.*, 2001 and Sikand *et al.*, 2000), drug therapy may be reserved for patients who are at high risk for CHD (Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults, 1993). It was shown that sesame and its lignans can reduce TC and LDL-C in hypercholesterolemic patients and postmenopausal women who have higher prevalence of hypercholesterolemia (Chang *et al.*, 2002).

### *Enhancement of vitamin E level*

The tocopherol and tocotrienol vitamers that comprise the vitamin E family are considered the most important lipophilic radical-quenching antioxidants in cell membranes. While their function is most often associated with the reduction of peroxy radicals, novel vitamer-specific roles for tocopherols in signal transduction and in the quenching of other reactive chemical species such as nitrogen dioxide and peroxynitrite are now being investigated (Brigelius-Flohe & Traber, 1999). While  $\alpha$ -tocopherol has attracted a lot of attention, recent studies indicate that several of these important roles may be specific to  $\gamma$ -tocopherol. For example it was shown that  $\gamma$ -tocopherol and its major metabolite, 2,7,8-trimethyl-2-(beta-carboxyethyl)-6-hydroxychroman ( $\gamma$ -CEHC), inhibit cyclooxygenase activity in stimulated macrophages and epithelial cells and therefore, reduces the synthesis of prostaglandin  $E_2$  ( $PGE_2$ ) (Jiang *et al.*, 2000, 2001). Moreover, in carrageenan-induced inflammation in male wistar rats, administration of  $\gamma$ -tocopherol (33 mg/kg) can reduce  $PGE_2$ , Leukotriene  $B_4$ , and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Jiang & Ames, 2003). It was demonstrated that administration of sesame oil and its lignans increase blood and tissue concentrations of  $\gamma$ -tocopherol without altering those of  $\alpha$ -tocopherol in rats (Yamashita *et al.*, 1992; Kamal-Eldin, *et al.*, 1995, 2000 and Ikeda *et al.*, 2002). The inclusion of unrefined sesame oil in the habitual diet of healthy women over a 4 week intervention period raised plasma level of  $\gamma$ -tocopherol by 42% without altering  $\alpha$ -tocopherol (Lemcke-Norojärvi *et al.*, 2001). In another study, plasma  $\gamma$ -tocopherol increased by 19% in volunteers only after 3 days of intervention with muffins containing sesame seed (equal to a daily dose of 35 mg sesamin and 13 mg sesamol) (Cooney *et al.*, 2001).

Sontag & Parker, (2002) suggested a cytochrome P450 4F2 mediated  $\omega$ -hydroxylation pathway for  $\gamma$ -tocopherol catabolism, which was inhibited by sesamin. It was also shown that the urinary excretion of  $\gamma$ -tocopherol metabolites was significantly lower in volunteers after the consumption of sesame oil muffins (Frank *et al.*, 2004a). These data suggest that sesamin can possibly inhibit the catabolism of  $\gamma$ -tocopherol, which results in its higher bioavailability observed in human and animal studies (Yamashita *et al.*, 1992; Kamal-Eldin, *et al.*, 1995, 2000; Ikeda *et al.*, 2002; Lemcke-Norojärvi *et al.*, 2001; Cooney *et al.*, 2001 and Sontag *et al.* 2002). Recently, Abe *et al.* (2005) demonstrated that dietary sesame seeds elevate  $\alpha$ -tocopherol concentration in rat brain. They showed that the concentration of  $\alpha$ -tocopherol in the brain of rats (Cerebrum, cerebellum, brain stem, and hippocampus) fed 50 mg  $\alpha$ -tocopherol/kg with sesame seeds was higher than that of the rats fed 500 mg  $\alpha$ -tocopherol/kg without sesame seed. These results suggest that the dietary sesame seeds are more useful than the intake of an excess amount of  $\alpha$ -tocopherol, for maintaining a high  $\alpha$ -tocopherol concentration and inhibiting lipid peroxidation in the various regions of the rat brain (Abe *et al.*, 2005).



### *Effect on lipid metabolism and inflammatory responses*

Diet containing sesame seeds (200 g/kg) was shown to increase both the hepatic mitochondrial and peroxisomal fatty acid oxidation rate in rats. Noticeably, peroxisomal activity levels were increased >3 times in rats fed diets containing sesame seeds than those fed a control diet without sesame seed. Sesame diet also significantly increased the activity of hepatic fatty acid oxidation enzymes including acyl-CoA oxidase, carnitine palmitoyltransferase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase (Sirato-Yasumoto *et al.*, 2001). In contrast, sesame diet lowered the activity of enzymes involving in fatty acid synthesis including fatty acid synthase, glucose-6-phosphate dehydrogenase, ATP-citrate lyase and pyruvate kinase (Sirato-Yasumoto *et al.*, 2001). Similar effects on hepatic fatty acid oxidation and synthesis were also observed when rats were fed diets containing pure sesamin (1) or episesamin (0.2%) for 15 day suggesting the possible involvement of sesame oil lignans in the induction of the observed changes in hepatic fatty acid metabolism (Kushiro *et al.*, 2002). Considering lower serum triacylglycerol observed in rats fed sesame seed diets, Sirato-Yasumoto *et al.* (2001) suggested that sesame seeds can possibly exert their triglyceride-lowering-effect through increasing fatty acid oxidation and reducing fatty acid biosynthesis in the liver. However, a 5-week intervention with 50 g pulverized roasted sesame seed in postmenopausal women (Wu *et al.*, 2006) or a 4-week intervention of 40 g roasted sesame seed in hypercholesterolemic patients (Chen *et al.*, 2005) did not alter the level of serum triglyceride.

Sesame seed lignans, such as sesamin (1), episesamin, sesamolin (2), sesaminol (3) as well as sesamol are known to inhibit the  $\Delta 5$ -desaturase activity, which is an enzyme in the biosynthesis of arachidonic acid from dihomo- $\gamma$ -linolenic acids *in vitro* (Shimizu *et al.*, 1991; Chavali & Forse, 1999) (**Figure 6**). Moreover, sesamin increases dihomo- $\gamma$ -linolenic acid and the ratio of eicosapentaenoic acid/ $\alpha$ -linolenic acid and decreased the ratio of arachidonic acid/ dihomo- $\gamma$ -linolenic acids in liver lipids of rats fed sesamin (0.5% w/w). Therefore, it has been suggested that sesamin inhibits the  $\Delta 5$ -desaturase activity of *n*-6 fatty acids but not that of *n*-3 fatty acids *in vivo* (Fujiyama-Fujiwara *et al.*, 1995; Mizukuchi *et al.*, 2003).

There are conflicting data concerning the arachidonic acid content of the liver lipids in rats fed sesamin, which can possibly be originated from different fatty acid profiles in the diet (Fujiyama-Fujiwara *et al.*, 1995; Kamal-Eldin *et al.*, 2000; Mizukuchi *et al.*, 2003). Wu *et al.* (2006) showed that consumption of 50 g roasted sesame seed for 5 weeks reduced the content of arachidonic acid in the serum lipids of postmenopausal women with no effect on dihomo- $\gamma$ -linolenic acids content. Arachidonic acid is the precursor of eicosanoids, which are a class of lipids including prostaglandins, thromboxanes and leukotrienes. The release of arachidonic acid from membrane phospholipids, which is the first stage in biosynthesis of eicosanoids, can occur as a result of tissue-specific stimuli. The released arachidonic acid then undergoes oxygenation by cyclooxygenase to yield prostaglandin H<sub>2</sub> (PGH<sub>2</sub>), which serves as precursor to other prostaglandins and thromboxanes (Mathews *et al.*, 2000). Chavali *et al.* (1998) showed that the

lipopolysaccharide-induced production of PGE<sub>2</sub> is lower and that of TNF- $\alpha$  is higher in mice fed a diet containing 0.25% sesamin. In addition, they showed that sesamol, a sesamol hydrolysis product, can reduce lipopolysaccharide-induced production of PGE<sub>2</sub> and interleukin 6 (IL-6) without increasing TNF- $\alpha$  (Chavali *et al.*, 1999). Proinflammatory mediators, such as PGE<sub>2</sub>, can influence the production of cytokines which mediate inflammatory responses during inflammation and infection (Pruimboom *et al.*, 1994). However, the reduction in PGE<sub>2</sub> in mice, exerted by sesamin and sesamol, poses different effect on interleukins and TNF- $\alpha$  possibly because of different mechanisms. Despite lack of differences in the levels of arachidonic acid, the PGE<sub>2</sub> levels were reported to be significantly lower in mice fed sesamin or sesamol supplemented diets. Therefore, Chavali *et al.* (1998, 1999) suggested that sesamin, sesamol or their metabolites decrease the activity of cyclooxygenase and the biosynthesis of prostaglandins.

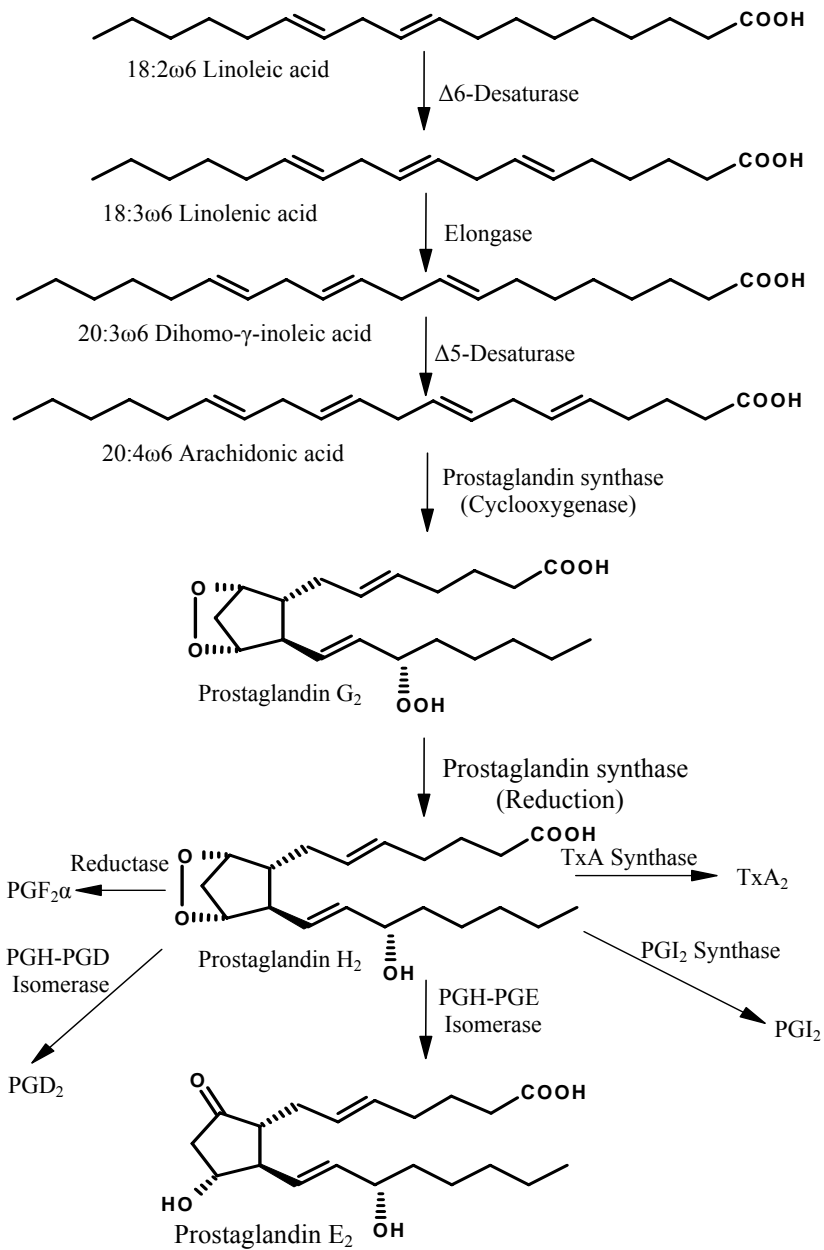


Figure 6. Biosynthesis of acachdonic acid and prostaglandins (E<sub>2</sub>) from linoleic acid. TxA<sub>2</sub>: Thromboxane A<sub>2</sub>, PG<sub>2</sub>: Prostaglandin G<sub>2</sub>, PH<sub>2</sub>: Prostaglandin H<sub>2</sub>, PE<sub>2</sub>: Prostaglandin E<sub>2</sub>, PGF<sub>2</sub> $\alpha$ : Prostaglandin PGF<sub>2</sub> $\alpha$ , PD<sub>2</sub>: Prostaglandin D<sub>2</sub>, PI<sub>2</sub>: Prostaglandin I<sub>2</sub>.

### *Bioactivity through conversion to mammalian lignans*

Consumption of sesame seed was shown to increase plasma and urinary excretion of enterolactone and enterodiols (Penalvo *et al.*, 2005; Coulman *et al.*, 2005). Epidemiological and laboratory studies have shown that high plasma and urinary concentration of enterolactone are inversely correlated with the risk of certain chronic disease, such as breast cancer (Adlercreutz *et al.*, 1982; Ingram *et al.*, 1997 and Pietinen *et al.*, 2001), prostate cancer (McCann *et al.*, 2005 and Hedelin *et al.*, 2006) and coronary heart disease (Vanharanta *et al.*, 1999; Vanharanta *et al.*, 2003 and Kilkkinen *et al.*, 2006).

Phytoestrogen, including mammalian lignans and isoflavones, are structurally and functionally comparable to estradiol-17 $\beta$  and are capable of producing estrogenic effect. Mammalian lignans bind to the estrogen receptors (ER) at lower level compare to the endogenous estrogen and may exert both estrogenic and antiestrogenic effects (Murkies *et al.*, 2005). It was shown that the concentration of enterolactone in the prostate fluid is significantly higher than their plasma level (Morton *et al.*, 1997). Estradiol-17 $\beta$  stimulates hepatic production of sex hormone-binding globulin. This protein binds both estrogens and androgens, regulating the levels of free hormones in the plasma. It was also shown that intervention with sesame seed in postmenopausal women can increase the level of sex hormone-binding globulin in serum (Wu *et al.*, 2006). However, whether this effect is exerted by sesame seed lignans or mammalian lignans produced from sesame seed lignans in gut is not clear. Moreover, it was shown that enterolactone upregulate LDL receptor activity in human hepatoma cell line (HepG2) (Owen *et al.*, 2004). This suggests another possible mechanism for cholesterol lowering effect of sesame seed and its lignans.

## Objectives

The objectives of this thesis included:

Isolation and characterization of major lignan glucosides in the defatted flour of sesame seeds (**Papers I, II**).

Method development for the analysis of lignan glucosides in sesame seeds (**Paper I**).

Quantification of lignan glucosides in the defatted sesame flour from a wide collection of sesame seeds and products (**Papers I, II, , IV**).

Quantification of lignans in the oil extracted from a wide collection of sesame seeds and products and commercial oils available in the market (**Papers III, IV**).

Identification of the major sesame lignan metabolite(s) in human urine after ingestion of a single dose of sesame oil (**Paper V**).

Development of NMR method for the quantification of the major sesamin metabolite in human urine after ingestion of a single dose of sesame oil (**Paper V**).

Investigation of the interactions between sesamin and phytosterols with regard to lipid metabolism using rats as a model (**Paper VI**).

## Materials and methods

### Isolation of sesame lignan glucosides from defatted sesame flour

Sesame seeds (with high concentration of lignans) were grinded and defatted with n-hexane to obtain defatted sesame flour (DSF), which was extracted with 80% ethanol to obtain a crude extract. This crude extract was then used for the isolation of lignan glucosides.

For the isolation of sesaminol triglucoside (13), the crude extract from DSF was dissolved in 25% methanol, applied on a Buckner funnel packed with Bondesil-C18 silica, and eluted with 25, 50, 75 and 100% methanol in series. The 50 and 75% fractions were pooled, dried and dissolved in chloroform/methanol (1:1, v/v) and applied on a silica column and the column was eluted with chloroform/methanol (1:1, v/v). Fractions were collected in 10 mL tubes and monitored by HPLC-DAD equipped with reversed-phase column to identify those tubes containing sesaminol triglucoside (**Paper I**).

For the isolation of the sesaminol diglucoside (14b), the crude extract was dissolved in methanol and used for thin-layer chromatography (TLC). The plates were developed using chloroform/methanol (1:1, v/v) and visualized by iodine vapour and  $R_f$  values for the different spots on the TLC plates were calculated. The spot ( $R_f$  0.65-0.72) was scraped, extracted and assigned as a new isomer of sesaminol diglucoside by LC-MS and NMR (**Paper I**).

For the isolation of sesamolol diglucoside (19), the crude extract from DSF was dissolved in chloroform/methanol (4:1, v/v) and applied on silica gel cartridges, which were eluted with chloroform/methanol (4:1, v/v). This was performed to increase the ratio of sesamolol diglucoside relative to more polar compounds, like sesaminol triglucoside, in the crude extract prior to separation and collection of sesamolol diglucoside by analytical HPLC-DAD equipped with reversed-phase column (**Paper II**).

### Characterization and identification of molecules

The structures of the compounds separated by different technique were assigned by nuclear magnetic resonance spectroscopy incorporating  $^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$ , COSY, TOCSY, HMBC, HSQC, ROESY and NOESY, HPLC-DAD and LC-MS equipped with electrospray ionization (ESI) and reversed phase column chromatography eluted with 10 mM acetic acid (pH 3) and acetonitrile (**Papers I, II, V**).

### Seeds, oil and products

In this thesis, two groups of sesame samples were analyzed for their contents of lignans and lignan glucosides.

*Group 1.* Sixty-five different sesame seed samples, bred, grown and harvested in Sesaco corporation nurseries in Texas, USA were used. The seeds had white (n=47), black (n=11), brown and yellow color (n=7). Seeds from semi-shattering (n=7) and non-dehiscent (n=29) plants were harvested when the plants were dry and the dehiscent types (shattering) (n=29) were harvested when half of the plants were dry. The plants were hand-selected, hand cut, put through a plot thresher and carefully cleaned to remove all foreign materials (**Papers I, II, III**).

*Group 2.* Eighteen different sesame seeds varying in colour (White, brown, black and mixed color) and in the presence (n=14) or absence of hull (n=4) were purchased from local market in different countries. Fourteen different products containing either sesame oil or sesame seeds including six sesame pastes (Tahini) varying in brands were acquired from different countries. Nine different sesame oil samples including virgin sesame oil (n=4) and roasted sesame oil (n=5) were purchased from local market of different countries (**Paper IV**).

#### *Quantification of oil-soluble lignans*

Sesame seeds and products were grinded and their oil was extracted using hexane/isopropanol (3:1, v/v). The solvent was then evaporated and the oil content was determined before it was analyzed for its contents of lignans (*vide infra*). The oil samples were dissolved in *n*-hexane and analyzed by an HPLC equipped with fluorescence detector and normal-phase column. The fluorescence detector was operated at an excitation wavelength of 296 nm and an emission wavelength of 324 nm. The sesamin, sesamol and  $\gamma$ -tocopherol contents of oil samples were then calculated using authentic external standards (**Papers III, IV**).

#### *Quantification of lignan glucosides*

The defatted flour after oil extraction was dried and used for the analysis of lignan glucosides. The defatted flour was first extracted with 85% ethanol containing naringenin as internal standard. The extraction was continued overnight with 70% ethanol after adjusting the extraction volume with water. This method was shown to provide higher recovery in comparison with either 70, 80 or 85% ethanol, possibly because of preventing enzymatic degradation or because of better extractability of lignan glucosides from matrix, when the extraction is performed with different mixtures of ethanol, which possess different polarity (**Papers I, II, IV**).

The quantities of sesaminol tri- and di-glucoside and sesamolol diglucoside were calculated from the peak areas at 290 nm against naringenin as internal standard using HPLC-DAD equipped with reversed-phase column. The response factor for sesaminol relative to naringenin was established as 0.345 and was assumed to be similar for sesamolol considering the similar extinction coefficients (Osawa *et al.*, 1985 and Katsuzaki *et al.*, 1994b) (**Papers I, II, IV**).

## Metabolism of sesame seed lignans by humans

After one week maintaining a diet low in lignans, overnight blank urine was collected from volunteers who participated in studies as follows:

In a pilot study, a 30-year-old man took commercial capsules containing sesamin (110 mg) and sesamol (6 mg). The urine was collected for 24 hours and used for the isolation of metabolite(s).

In the main study, volunteers (n=6) ate muffins baked with sesame oil containing sesamin (180 mg) and sesamol (71 mg). The treatment urine was then collected for four 12-hour-intervals. The samples were at -20°C until the analysis.

The urine samples were deconjugated by  $\beta$ -glucuronidase/sulphatase and extracted with chloroform. The chloroform extracts were dried, dissolved in methanol and separated by analytical HPLC-DAD equipped with reversed-phase column. A peak (RT=37.5 min) with characteristic lignan spectra was collected from the chloroform extract of treatment urine using the analytical HPLC. This peak was then characterized using NMR and LC-MS (**Paper V**).

### *Quantification of major urinary metabolite(s) by <sup>1</sup>H-NMR and HPLC*

The amount of the sesame lignan metabolite in the chloroform extract of deconjugated urine from the volunteers was quantified by NMR. Portions of the chloroform extract were dried and dissolved in CD<sub>3</sub>OD and analyzed by <sup>1</sup>H-NMR. Signals corresponding to protons of the methylenedioxyphenyl group (MDP) ( $\delta$  5.91) in <sup>1</sup>H-NMR spectra of the treatment urine were used for the quantification of the metabolite in the chloroform extracts of urine against authentic sesamin solution in CD<sub>3</sub>OD. Sesamin has two MDP groups in its molecule. Therefore, it was assumed that sesamin has twice the NMR response in similar molar solution as its catechol metabolites. The methyl group from residual methanol in CD<sub>3</sub>OD was used as internal standard and the ratio between the areas of MDP protons ( $\delta$  5.91) and the methyl group signals from residual methanol ( $\delta$  3.31) were used to draw the calibration curve (**Paper V**). The sesamin equivalent of metabolite was also analyzed by HPLC-DAD and the results were compared with those from NMR quantification.

### *Pilot study on the effect of acidic condition on sesaminol triglucoside*

Defatted sesame flour was extracted by 80% ethanol and the extract was dried, dissolved in water (pH=0.8-1.5, HCl) and incubated at 37°C for 1, 3 and 20 hours. The samples were analyzed by LC-MS and HPLC-DAD and compared with a standard solution of sesaminol triglucoside subjected to similar treatment to investigate the effect of acidic incubation on sesaminol triglucoside in aqueous solution.



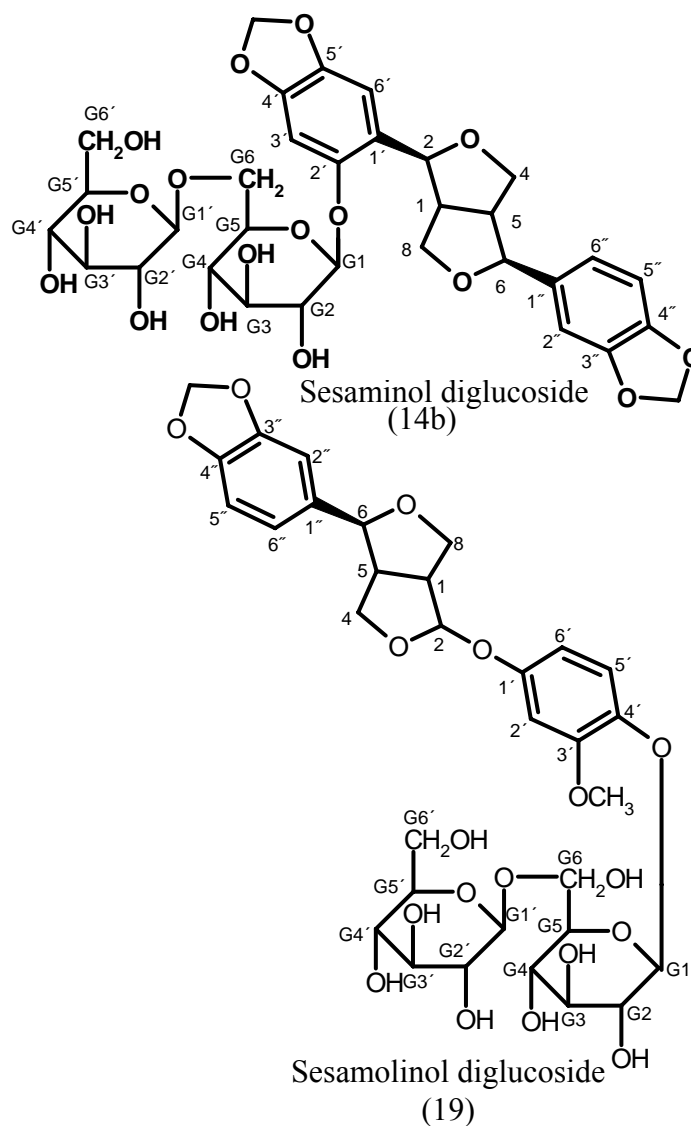
### **Interactions between sesamin and phytosterols with regard to lipid metabolism in rats**

Sprague Dawley rats were randomly assigned to 4 groups (n=6) with similar body weights. The experiment was performed in a 2×2 factorial design. Rats were fed diets containing sesamin (0 and 1.6 g/kg diet) and phytosterols (0.6 and 1.1 g/kg diet) for four weeks. At the end of the experiment, the rats were fasted for 12 hours before being killed and their blood (plasma) and liver were collected and frozen. Plasma samples were analyzed for tocopherols and cholesterol and liver lipids were extracted and analyzed for tocopherols, cholesterol and fatty acid profile (**Paper VI**).

## Results and discussion

### Characterization of new lignan glucosides (Papers I, II)

Two new sesame lignan glucosides (**Figure 7**) were isolated and characterized in this thesis by NMR (**Table 2**) and LC-MS (**Table 3**).



*Figure 7.* Sesaminol diglucoside and sesamolol diglucoside isolated from sesame seeds.

Table 2. <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectral data of sesaminol diglucoside and sesaminol diglucoside isolated from sesame seeds.

Position	Sesaminol Diglucoside		Sesaminol Diglucoside	
	$\delta_H$	$\delta_C$	$\delta_H$	$\delta_C$
1	3.05	55.3	3.33 m	53.9
2	5.21 d, J=4.7	82.6	5.65 s	108.3
4	3.89 dd, J=4.2, 9.1 (a)	72.1	4.00 dd, J=1.1, 9.2 (a)	70.3
4	4.22 dd, J=6.4, 9.1 (b)	-	4.15 dd, J=5.9, 9.2 (b)	-
5	3.00 m	55.5	2.98 m	54.3
6	4.66 d, J=5.4	86.6	4.46 d, J=7.0	88.5
8	4.09 dd, J=4.4, 9.1 (a)	73.9	4.40 t <sup>b</sup> , J=9.2, 9.2 (a)	71.7
8	4.31 dd, J=7.3, 9.1 (b)	-	3.68 dd, J=5.1, 9.2 (b)	-
1'	-	125.6	-	154.8
2'	-	150.5	6.73 d, J=2.5	103.8
3'	6.93 s	99.5	-	151.8
4'	-	144.2	-	142.9
5'	-	148.6	7.12 d J=8.8	119.4
6'	6.85 s	105.7	6.62 dd, J=2.5, 8.8	109.5
1''	-	136.4	-	136.0
2''	6.87	107.1	6.9 d, J= 1.7	107.2
3''	-	149.0	-	149.5
4''	-	148.6	-	148.7
5''	6.77	108.8	6.79 d, J= 8.1	108.7
6''	6.84	120.4	6.86 dd, J= 1.7, 8.1	120.3
-O-CH <sub>2</sub> -O-'	5.91	101.9	5.94 s	101.8
-O-CH <sub>2</sub> -O-''	5.90	102.0	-	-
-O-CH <sub>3</sub>	-	-	3.84 s	56.6
G1	4.85 <sup>a</sup>	102.9	4.80 d, J= 7.3	103.3
G2	3.46	74.86	3.47	74.8
G3	3.68	77.0	3.43	77.4
G4	3.44	71.1	3.40	71.1
G5	3.45	77.9	3.58	77.6
G6a	4.15 dd, J=1.9, 11.5	70.0	4.13 dd, J= 1.8, 11.7 (a)	69.3
G6b	3.83 m	-	3.82 dd, J= 2.6, 11.7 (b)	-
G1'	4.35 d, J=7.7	104.5	4.37 d, J= 7.7	104.2
G2'	3.24 dd, J=8.1, 7.7	74.9	3.19	74.9
G3'	3.36	77.7	3.30	77.6
G4'	3.31	71.5	3.28	71.5
G5'	3.21	77.7	3.20	77.7
G6'a	3.85 m	62.7	3.87 m (a)	62.7
G6'b	3.64 m	-	3.66 m (b)	-

<sup>a</sup> Because of virtual higher order, the coupling constant could not be read. <sup>b</sup> Resulting from the overlap of two doublets.

### *Sesaminol diglucoside (14b) (Figure 7)*

H-1 ( $\delta$  3.05) appeared as a multiplet due to coupling with H-8a, H-8e and H-2. Similarly, H-5 ( $\delta$  3.00) appeared as a multiplet due to coupling with H-4a, H-4e and H-6. These results were confirmed by COSY. HMBC confirmed the coupling between H-2 ( $\delta$  5.21) and C-8 ( $\delta$  73.9) and between H-6 ( $\delta$  4.66) and C-4 ( $\delta$  72.1). HMBC also confirmed coupling between H-1 and C-1' ( $\delta$  125.6), between H-2 and C-5' ( $\delta$  148.6), between H-5 and C-1'' ( $\delta$  136.4) and between H-6 and C-6'' ( $\delta$  120.4) and C-2'' ( $\delta$  107.1).  $^1\text{H}$  NMR showed coupling between H-6'' and H-2'' ( $J=1.5$  Hz). The position of C-4'' ( $\delta$  148.6) was assigned from coupling with H-6'' in HMBC. The aromatic carbons were assigned from coupling to corresponding hydrogen in HSQC. The glycosylation at C-2' ( $\delta$  150.5) was confirmed by a cross peak with the anomeric proton (G1,  $\delta$  4.85) and a down-field shift in comparison with C-2'' ( $\delta$  107.1). HMBC showed a cross peak between the anomeric proton (G1',  $\delta$  4.35) and C-G6 ( $\delta$  70.0) indicating a 1 $\rightarrow$ 6 bond between two sugars. Moreover, ROESY and NOESY spectra indicated a cross peak between the anomeric proton (G1',  $\delta$  4.35) and G6a ( $\delta$  3.83) and G6b ( $\delta$  4.15), which confirmed the result obtained by HMBC concerning the 1 $\rightarrow$ 6 bond between the two sugars. This sugar linkage (1 $\rightarrow$ 6) was different from that in other sesaminol diglucoside isomer (14a) isolated and characterized by Katsuzaki *et al.* (1994b), in which the sugar residue had 1 $\rightarrow$ 2 bond. The coupling constant for the anomeric protons G1' ( $\delta$  4.88,  $J=7.70$  Hz) confirmed the  $\beta$ -configuration of these proton (Katsuzaki *et al.*, 1994b), however for G1 ( $\delta$  4.85), because of virtual higher order, the coupling constant could not be read (**Paper I**). The structure of sesaminol triglucoside was assigned in the same way as for sesaminol diglucoside. Moreover, a peak, which was assigned as P3 on the HPLC-DAD chromatogram and co-eluted with sesaminol diglucoside at  $R_f=0.72-0.75$  on TLC plates was collected using analytical HPLC and analyzed by LC-MS. It had similar ionization at positive mode with sesaminol diglucoside, however the pattern of ionization was different in negative mode **Table 3**. In addition, the UV spectrum of P3 was similar to sesaminol diglucoside **Table 3**. Therefore, P3 may be the sesaminol diglucoside (14a), which was previously isolated and characterized by Katsuzaki *et al.* (1994b).

### *Sesamolinal diglucoside (19) (Figure 7)*

H-1 ( $\delta$  3.3) appeared as a multiplet due to coupling with H-8a, H-8b, H-5 and H-2, while H-5 ( $\delta$  2.98) appeared as a multiplet due to coupling with H-4a, H-4b, H-1 and H-6, as confirmed by COSY. HMBC confirmed coupling between H-2 ( $\delta$  5.65) and C-8 ( $\delta$  71.7), between H-6 ( $\delta$  4.46) and C-8 ( $\delta$  71.7), between H-4a ( $\delta$  4.0) / H-8a ( $\delta$  4.40) and C-2 ( $\delta$  108.3), between H-8a ( $\delta$  4.40) and C-6 ( $\delta$  88.5), and between H-4a ( $\delta$  4.0) / H-4b ( $\delta$  4.15) and C-6 ( $\delta$  88.5). HMBC also confirmed the coupling between H-6 ( $\delta$  4.46) and C-6'' ( $\delta$  120.3) and C-2'' ( $\delta$  107.2).  $^1\text{H}$ -NMR showed *meta*-coupling between H-6'' and H-2'' ( $J=1.7$  Hz) and *ortho*-coupling between H-6'' and H-5'' ( $J=8.1$  Hz). The position of C-4'' ( $\delta$  148.7) was assigned from the coupling with H-2'' ( $\delta$  6.9), and the position of C-3'' ( $\delta$  149.5) was assigned from the coupling with H-5'' ( $\delta$  6.79) by HMBC. The aromatic carbons were assigned from the coupling to the corresponding hydrogen by HSQC. The position of the methoxyl group of the aromatic ring was confirmed

from a cross peak between its methoxy protons ( $\delta$  3.84) and C-3' ( $\delta$  151.8). Glycosylation at C-4' ( $\delta$  142.9) was shown by a cross peak with the anomeric proton (G1,  $\delta$  4.81) by HMBC. The NOESY spectrum indicated a cross peak between this anomeric proton (G1,  $\delta$  4.81) and H-5' ( $\delta$  7.12), confirming the position of glycosylation on C-4'. An up-field shift in position C-4' ( $\delta$  142.9) in comparison with position C-4'' ( $\delta$  148.7) indicated the presence of two oxygen atoms in a *para* position around the benzene ring. Moreover, there were down-field shifts in C-2 ( $\delta$  108.3) and C-1' ( $\delta$  154.8) in comparison with C-6 ( $\delta$  88.5) and C-1'' ( $\delta$  136.0). These results confirmed the presence of oxygen between the furofuran and benzene rings (**Paper II**). HMBC showed a cross peak between the anomeric proton (G1',  $\delta$  4.37) and C-G6 ( $\delta$  69.3), indicating a 1 $\rightarrow$ 6 bond between the sugars. Moreover, the NOESY spectrum indicated a cross peak between the anomeric proton (G1',  $\delta$  4.37) and G6b ( $\delta$  4.13), confirming this 1 $\rightarrow$ 6 bond between the sugars (**Paper II**). This (1 $\rightarrow$ 6) sugar linkage is similar to that reported for sesaminol diglucoside discussed above.

Table 3. Characteristic studies of defatted sesame flour extract by TLC, HPLC-DAD and LC-MS.

Compound	TLC <sup>a</sup> R <sub>f</sub>	HPLC <sup>b</sup> t <sub>R</sub> (min)	UV absorption <sup>b</sup> (λ, nm)	LC-MS <sup>c</sup> positive mode	LC-MS <sup>c</sup> negative mode
Sesaminol Triglucoside	0.36-0.41	19.0	238, 291	[S <sub>3</sub> G+Na] <sup>+</sup> =879.2	[S <sub>3</sub> G-H] <sup>-</sup> =855.2
Sesaminol Diglucoside	0.65-0.72	25.6	238, 292	[S <sub>2</sub> G+Na] <sup>+</sup> =717.1	[S <sub>2</sub> G+CH <sub>3</sub> COO] <sup>-</sup> =753.2
P3	0.72-0.75	27.0	239, 293	[S <sub>2</sub> G+Na] <sup>+</sup> =717.1	[S <sub>2</sub> G-H] <sup>-</sup> =693.2

<sup>a</sup>Pre-coated silica gel 60 TLC plates developed by chloroform/methanol (1:1, v/v). <sup>b</sup>The eluents used were: (A) 0.01 M phosphate buffer (pH 2.8) containing 5% Acetonitrile and (B) Acetonitrile. The elution conditions were: 0-5 min (15% B), 30 min (30% B), 40-50 (70% B) and the flow rate was 1.0 mL/min. <sup>c</sup>Electrospray ionization. The eluents used were: (A) 10 mM acetic acid (pH 3) and (B) Acetonitrile. The elution conditions were 0-5 min (20% B), 30-50 min (70% B) and the flow rate was 0.4 ml/min. At the interface, the drying gas was set to 9 L/min, the nebulizer pressure was 30 psi, the drying gas temperature was 350 °C and the scanning range was 300-1000 m/z. S<sub>3</sub>G: Sesaminol triglucoside, S<sub>2</sub>G: Sesaminol diglucoside.

## Development and validation of the analytical method for lignan glucosides (Paper I)

The effects of ethanol percentage, extraction time, cake to solvent ratio and temperature on the extraction efficiency of lignan glucosides were investigated in this thesis, with more emphasis on sesaminol triglucoside. The recovery of sesaminol triglucoside depended on ethanol concentration, where 70% (9.9 mg/g of DSF) and 80% (10 mg/g of DSF) provided higher recovery of sesaminol triglucoside compared with 50% (7.6 mg/g of DSF), 60% (9.3 mg/g of DSF), and 90% (3.3 mg/g of DSF) ( $p < 0.01$ ). The recovery was increased over the time (from 15 h to 72 H) when DSF was extraction with 85% ethanol. However, the prolongation of the extraction time, from 15 h (9.8 mg/g of DSF) to 72 h (9.39 mg/g of DSF), caused a 4.2% decrease in the yield of sesaminol triglucoside when DSF was extracted with 70% ethanol ( $p < 0.001$ ), which can possibly be explained by degradation. To gain the mutual benefit of higher recovery and less degradation, a method was developed based on extraction with 85% ethanol for 5 h followed by 70% ethanol for 10 h. This extraction method provided better yield (10.4 mg/g of DSF) in comparison with extraction with either 70% (9.9 mg/g of DSF) or 85% ethanol (5.9 mg/g of DSF) ( $p < 0.001$ ). The recovery increased significantly ( $n = 5$ ,  $p < 0.01$ ,) when the DSF to solvent ratio decreased from 0.8 g/10 mL (10.05 mg/g of DSF) to 0.25 g/10 mL (10.5 mg/g of DSF), but no significant difference in recovery was found between 0.25 g/10 mL (10.5 mg/g of DSF) and 0.5 g/10 mL (10.4 mg/g of DSF). Therefore, considering the small coefficient of variation ( $CV = 0.6\%$ ) when 0.5 g/10 mL was used, this was chosen as a proper ratio. To investigate the effect of extraction temperature on the extractability of sesaminol triglucoside, DSF was extracted at 20, 37, and 55°C. Statistical analysis revealed no difference between the different temperatures used and therefore, room temperature was chosen. Similar effects of ethanol percentage, extraction time and temperature were observed on the recovery of sesaminol diglucoside and sesamolinal diglucoside (**Paper I**).

The effect of the solid matrix on the recovery of internal standard, naringenin, was investigated by spiking different amounts of DSF (0.25-0.8 g/10 mL) with different concentrations of internal standard (50-200 µg/mL). Analysis of variance indicated no significant effect of DSF matrix on the recovery of naringenin under the investigated range. The precision of the method was determined by analyzing sesaminol triglucoside in DSF in six different days. Each day, sesame cake was extracted and analyzed by HPLC using the method described. The intra-assay precision (within day variation) ranged from 0.6 to 2.8% with the average of 1.6%, and the inter-assay precision (between days variation) was 2.8% (**Paper I**).

Using sesaminol triglucoside standard, the detection limits of the method were determined as 10 mg/100 g of DSF for sesaminol triglucoside and 8 mg/100 g of DSF for sesaminol diglucoside and sesamolinal diglucoside using a signal-to-noise ratio of 3. The concentration of lignan glucosides having a peak height 10 times higher than noise was considered as the limit of quantification (40 mg/100 g of DSF for sesaminol triglucoside and 32 mg/100 g of DSF for sesaminol diglucoside and sesamolinal diglucoside). Linearity was confirmed between the

lowest and highest concentrations of sesaminol used in the standard curve (9 and 435 µg/mL, respectively) (**Paper I**). In **paper III** the lignan glucosides analyzed in this thesis were reported as their aglycones equivalents, sesaminol and sesamolinal. The aglycones equivalent of detection limit and quantification limit were 2.2 and 8.8 µg/mL respectively for both sesaminol and sesamolinal.

### **Validation of the analytical method for oil-soluble lignans**

Sesamin and sesamolinal were analyzed by HPLC equipped with a fluorescence detector and a silica column as mentioned above. For sesamin and sesamolinal, the oil-soluble lignans, the detection limits were determined 5 mg/100 g oil and 10 mg/100g oil respectively using standard solutions and a signal-to-noise ratio of 3. The concentration of sesamin (15 mg/100g oil) and sesamolinal (35 mg/100g oil) having peak height 10 times higher than noise were considered as the limit of quantification. Linearity was confirmed between quantification limit and highest concentration of sesamin and sesamolinal used in standard solutions (75 µg/mL) (**Paper III**).

### **Variation of lignans and lignan glucosides in sesame seeds and products**

The first group of samples was newly bred sesame seeds (n=65) from plants with different capsule types, which were grown in controlled condition. Sesaminol triglucoside, sesaminol diglucoside and sesamolinal diglucoside were analyzed in DSF and their aglycone equivalents in the seeds were calculated and presented in **Table 4 (Papers I, II, III)**. The sesame oil-soluble lignans, sesamin and sesamolinal, were also analyzed and their contents in seeds are presented in **Table 4 (Paper III)**. Total sesaminol, both in triglucoside and diglucoside form, ranged 16-720 mg/100 g seeds (mean±SD 153±317 mg/100 g seed) and was higher than what Ryu *et al.* (1998) had previously reported in 25 cultivars. Sesamolinal, which was originally analyzed in glucoside form, ranged from undetectable to 124 mg/100 g seed (31±53 mg/100 g seed). The analysis of variance indicated no significant difference between the content of lignan glucoside in black and white seeds (**Papers I, II, III**). Sesamin content ranged 7-712 mg/100g seeds (163±141 mg/100g seed) and sesamolinal content ranged 21- 297 mg/100g seed (101±58 mg/100g seed). The analysis of data indicated a wider range of sesamin content than what was reported by Tashiro *et al.* (1990) but Hemalatha and Ghafoorunissa (2004) reported higher sesamin and sesamolinal contents than what is reported in this thesis. The low sesamin and sesamolinal contents found in this study were not observed by previous investigators (Kamal-Eldin & Appelqvist, 1994; Tashiro *et al.*, 1990; Hemalatha & Ghafoorunissa, 2004; Yermanos *et al.*, 1972; Fukuda *et al.*, 1988a and Beroza *et al.*, 1995). Statistical analysis revealed no significant difference between sesamin and sesamolinal contents of white and black seeds (**Paper III**).

The second group of sesame seeds and products were purchased from different markets (**paper IV**). The levels of lignans in 14 sesame seeds varied considerably. Sesamin (167-804 mg/100g seeds) was the major lignan followed by sesamolinal



(48-279 mg/100g seeds) and sesaminol (32-298 mg/100g seeds) while sesamolol was found in much lower concentrations (tr.-58 mg/100g seeds). The variations in the levels of sesamin, sesamolol and sesaminol and sesamolol were within the wide range reported in previous publications (Kamal-Eldin & Appelqvist, 1994; Tashiro *et al.*, 1990; Hemalatha & Ghafoorunissa, 2004; Yermanos *et al.*, 1972; Fukuda *et al.*, 1988a and Beroza *et al.*, 1995) and what was already seen in this thesis for the first group of samples (**Papers I, II, III**). The levels of lignans in 4 samples of dehulled seeds was also within the previously reported ranges suggesting that sesame lignans and lignan glucosides are not exclusively located in the hull. Dehulled sesame seeds are commercially interesting since they are used in food products including hamburger buns, tahini and halva. The level of lignans in crackers, biscuits and bread depends on the degree of incorporation of sesame seeds or oil. Virgin sesame oils and sesame oils extracted from roasted seeds contain comparable levels of sesamin and sesamolol, the native sesame seed lignans.

The results obtained from the analysis of first and second groups of samples (**Papers I, II, III, IV**) suggest that sesame seeds may be as a rich source of lignans as flaxseed. Since sesame seeds are consumed as food to much larger extent than flaxseed, they and especially their oil might provide the richest sources of dietary lignans. It is, thus, important to include the intake of sesame seeds and products in studies aiming to estimate dietary intake of phytoestrogens. However, the wide variability in the types and levels of lignans in sesame seeds and oils poses a great challenge to their incorporation in databases.

Table 4. Variation in the contents of oil (%), sesamin, sesamolin , sesaminol and sesamolinal in 65 different sesame seed samples (group 1) (mg/100 g seed, fresh weight).

	Oil	Sesamin	Sesamolin	Sesaminol	Sesamolinal	Total
Plant with shattering capsules (n=29)						
Range	33-49	13-712	29-297	16-437	ND-110	258-1159
Mean	44	206	125	222	38	590
Plants with Semi-Shattering Capsules (n=7)						
Range	41-51	12-380	44-160	110-425	18-97	499-675
Mean	46	146	119	269	52	587
Plants with Non-Dehiscent Capsules (n=29)						
Range	38-48	7-390	21-167	134-720	9-124	433-862
Mean	44	124	73	424	68	688
All Sesame Seeds (n=65)						
Range	33-51	7-712	21-297	16-720	0-124	224-1148
Mean	44	163	101	153	31	581

### *Effect of shattering resistance on the lignan contents of sesame seeds* **(Paper III)**

The effect of capsule type on the lignan contents of sesame seeds was studied (**Table 4**). Seed samples from plants with semi-shattering (n=7) and non-dehiscent (n=29) capsules were analyzed for the first time in this thesis and their contents of lignans were compared with seeds from plants with shattering capsules (n=29). The analysis of variance showed that there was no difference between the sesamin content in seeds from plants with shattering and non-dehiscent capsules but the content of sesamolol was significantly higher in seeds from plants with shattering capsules (mean±SD 125±67 mg/100g) than in seeds from plants with non-dehiscent capsules (73±37 mg/100g,  $p<0.001$ ). Conversely, the contents of sesaminol and sesamolol were higher in the seeds from plants with non-dehiscent capsules (424±131 and 68±30 mg/100g seed, respectively) than their contents in the seeds from plants with shattering capsules (222±112 and 38±26 mg/100g seed) respectively ( $p<0.001$ ). The total lignan content (sum of sesamin, sesamolol, sesaminol and sesamolol) was higher in seeds from plants with non-dehiscent capsules (688±10 mg/100g seed) than the seeds from plants with shattering capsules (590±19 mg/100g seed,  $p<0.05$ ) (**Paper III**). It was shown that lignan formation is developmentally regulated and depends on the stage of seed maturity. Therefore, the contents and ratio of lignans were changed by maturation (Jiao *et al.*, 1998; Ono *et al.*, 2006). This can possibly explain the differences observed in the contents of lignans in seeds from shattering and non-dehiscent capsules, which were harvested in different times. Higher sesamolol in seeds from shattering capsules, which was reported in this thesis, is in line with Jiao *et al.* (1998) that younger seeds had higher conversion of pinoreosinol to sesamolol, while higher content of sesaminol observed in seeds from non-dehiscent capsules is in line with Ono *et al.* (2006) that more mature seeds contain higher sesaminol. Sesame seeds with shattering capsules are the most utilized today and are mainly grown in Asian and African countries. The plants are harvested by hand before drying of the capsules to prevent the loss of seeds on the field following capsule shattering. Non-dehiscent plants can be mechanically harvested on the field when the plant is dry providing economical advantages over the traditional, capsule-shattering plants.

### *Correlation of lignans and lignan glucosides*

The contents of sesamin and sesamolol in seeds were positively and significantly correlated ( $R^2=0.69$ ,  $p<0.001$ ) as were the contents of the glucosylated lignans, sesaminol and sesamolol ( $R^2=0.53$ ,  $p<0.001$ ). The correlation between sesamin and sesamolol was stronger for black ( $R^2=0.77$ ) than for the white seeds ( $R^2=0.66$ ). The oil-soluble lignans, sesamin and sesamolol, correlated negatively with the glucosylated lignans, sesaminol and sesamolol. It is likely that the positive correlation between sesamin and sesamolol is related to similar pathways in their biosynthesis and that the negative correlation with the glucosylated lignans is related to the activity of some enzyme(s) in the biosynthetic pathway. The few studies on the biosynthesis of sesamin and sesamolol do not provide information about pathways to the glucosylated lignans (Marchand *et al.*, 1997; Kato *et al.*, 1998 and Jiao *et al.*, 1998) (**Paper III**).

## Human metabolism and urinary excretion of sesame oil lignans (paper V)

Sesamin, the major sesame oil lignan, was shown to cause an increase in  $\gamma$ -tocopherols in the plasma and liver and a reduction in liver cholesterol of rats contrary to secoisolariciresinol diglucoside, the major lignan glucoside in flaxseed (Kamal-Eldin *et al.*, 1995; Frank *et al.*, 2004b). The differences between the physiological effects of sesamin and those of secoisolariciresinol diglucoside, while both of them are converted to mammalian lignans by intestinal microflora, indicate that sesamin exerts its effects possibly through its intact molecule or different metabolic products. In addition, the cleavage of methylenedioxyphenyls in sesamin to form catechol or methoxy catechol was already shown (Nakai *et al.*, 2003; Liu *et al.*, 2006). Therefore, the aim of this study was to investigate the pattern of urinary metabolite(s) of sesame oil lignans in humans and to develop and validate a new quantitative NMR method to analyze the metabolite(s) in urine.

### *Separation of the major sesamin metabolite*

Blank urine and the urine samples collected after sesamin intervention were incubated with deconjugating enzyme ( $\beta$ -glucuronidase/sulphatase) or acetate buffer without enzyme, extracted with chloroform and analyzed by HPLC. Comparison of the HPLC chromatograms of blank and treatment urine samples in the pilot study revealed that peaks eluting at retention time 35-42 min appeared only after deconjugation suggesting the presence of conjugated metabolites in urine. The chloroform extract of the deconjugated sesamin treatment urine possessed a peak (RT=37.5 min) with characteristic UV spectrum of lignans ( $\lambda_{\text{max}}$ =234, 285 nm), which was not observed in the extract of deconjugated blank urine. This peak was collected from the HPLC effluent and characterized by NMR and LC-MS.

### *Structural characterization of major sesamin metabolite in human urine*

The peak collected from HPLC as described above was analyzed by NMR and its  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra data are presented in **Table 5**. H-1 ( $\delta$  3.11) appeared as a multiplet due to coupling with H-8a, H-8b, H-5 and H-2 while H-5 ( $\delta$  3.09) appeared as a multiplet due to coupling with H-4a, H-4b, H-1 and H-6 as confirmed by COSY. HMBC showed the coupling between H-6" ( $\delta$  6.84) / H-2" ( $\delta$  6.87) and C-6 ( $\delta$  87.1).  $^1\text{H}$  NMR showed *meta*-coupling between H-6" and H-2" ( $J=1.5$  Hz) and *ortho*-coupling between H-6" and H-5" ( $J=8.1$  Hz). The position of C-4" ( $\delta$  148.7) was assigned from coupling with H-2" ( $\delta$  6.87) and H-6" ( $\delta$  6.84) and the positions of C-3" ( $\delta$  148.7) and C-1" ( $\delta$  136.2) were assigned from coupling with H-5" ( $\delta$  6.77) in HMBC. Moreover, HMBC showed coupling between protons ( $\delta$  5.91) and C-4" ( $\delta$  148.7) confirming the presence of MDP group on this aromatic ring. On the other aromatic ring of the molecule, HMBC

showed the coupling between H-6' ( $\delta$  6.69) / H-2' ( $\delta$  6.8) and C-2 ( $\delta$  87.1).  $^1\text{H}$  NMR showed *meta*-coupling between H-6' and H-2' ( $J=1.8$  Hz) and *ortho*-coupling between H-6' and H-5' ( $J=8.1$  Hz). The position of C-4' ( $\delta$  145.7) was assigned from coupling with H-2' ( $\delta$  6.8) and H-6' ( $\delta$  6.69) and the positions of C-3' ( $\delta$  146.4) and C-1' ( $\delta$  133.5) were assigned from coupling with H-5' ( $\delta$  6.74) in HMBC. The presence of hydroxyl groups on this aromatic ring caused an up-field shift in positions C-3' and C-4' and a down-field shift in positions C-2' and C-5' in comparison with the corresponding positions on the other aromatic rings possessing MDP group. The aromatic carbons were assigned from coupling to corresponding hydrogen in HSQC. The molecular weight of isolated metabolite was further corroborated using LC-MS, where it had a  $[\text{M-H}]^-$  ion at  $m/z$  341.2 with electrospray ionization (ESI). Thus, the structure of the sesamin metabolite was established as (1*R*,2*S*,5*R*,6*S*)-6-(3,4-dihydroxyphenyl)-2-(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo-[3,3,0]octane by NMR and mass spectroscopy (**Figure 8**).

Table 5.  $^1\text{H}$ -NMR (600 MHz) and  $^{13}\text{C}$ -NMR (199 MHz) spectral data of sesamin metabolite in human urine.

Position	Chemical shift	
	$\delta_{\text{H}}$	$\delta_{\text{C}}$
1	3.11 m	55.1
2	4.65 d, $J=4.8$	87.1
4	3.82 m	72.4
	4.20 m	
5	3.09 m	55.1
6	4.7 d, $J=5.1$	87.1
8	3.83 m	72.4
	4.23 m	
1'	-	133.5
2'	6.8 d, $J=1.8$	114.2
3'	-	146.4
4'	-	145.7
5'	6.7 d, $J=8.1$	116.2
6'	6.69 dd, $J=1.8, 8.1$	118.5
1''	-	136.2
2''	6.87 d, 1.5	107.3
3''	-	148.7
4''	-	148.7
5''	6.77 d, 8.1	108.7
6''	6.84 dd, 8.1, 1.5	120.1
-O-CH <sub>2</sub> -O-	5.91 s	101.8

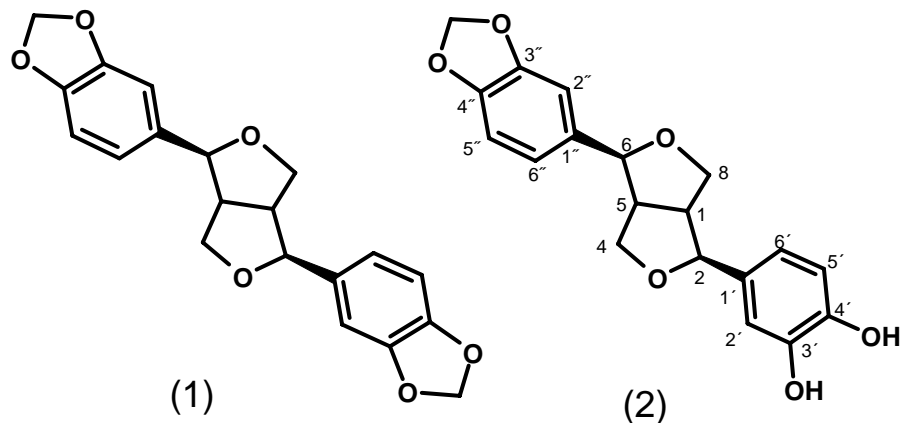


Figure 8. Structure of (1) sesamin and (2) its urinary catechol metabolite characterized in this study.

#### *Quantitative NMR technique*

A NMR based method for the quantification of sesamin metabolite in the deconjugated urine extract using signal corresponding to MDP ( $\delta$  5.91) was developed in this thesis. The validation data was presented as follows:

Experiments were performed to confirm that the peak corresponding to the MDP proton ( $\delta$  5.91) in  $^1\text{H-NMR}$  spectra of the urine extracts belong to the sesame lignan metabolites. Comparison between  $^1\text{H-NMR}$  spectra of the extract of blank urines and those of urines collected after sesamin intervention showed that the spectra contained MDP protons ( $\delta$  5.91) only after the intervention (**Figure 9**). Moreover, when the HPLC peak corresponding to the major sesamin metabolite was collected in a separate fraction (37-38 min) (**Figure 9**) this fraction contained protons with chemical shift at  $\delta$  5.91 in its NMR spectrum. The two other fractions collected between 0-37 min and between 38-70 min did not contain any signal with the chemical shift at  $\delta$  5.91. This provided evidence that protons on the MDP group of the metabolite (RT 37.5 min), which was characterized in this study, account for the signal at  $\delta$  5.91 in the  $^1\text{H-NMR}$  spectrum of urine extracts.

The detection limit of the NMR method for the metabolite was determined as 0.5  $\mu\text{g/mL}$  of urine using a signal-to-noise ratio of 3 and the limit of quantification was determined as 2  $\mu\text{g/mL}$  of urine using a signal-to-noise ratio of 10. Linearity was confirmed between the lowest and highest concentrations of sesamin used in calibration curve (0.1-0.4  $\mu\text{mol}$  sesamin dissolved in 0.5 mL  $\text{CD}_3\text{OD}$ ). The sesamin equivalents of the metabolite in the urine extracts were determined using

HPLC-DAD (285 nm) and the results were compared with the data obtained by the NMR method. The following correlation was obtained:  $Y=1.16X+4.06$ ,  $R^2=0.91$  where, Y and X were the concentrations ( $\mu\text{g/mL}$  of urine) of the metabolite as quantified by NMR and HPLC, respectively.

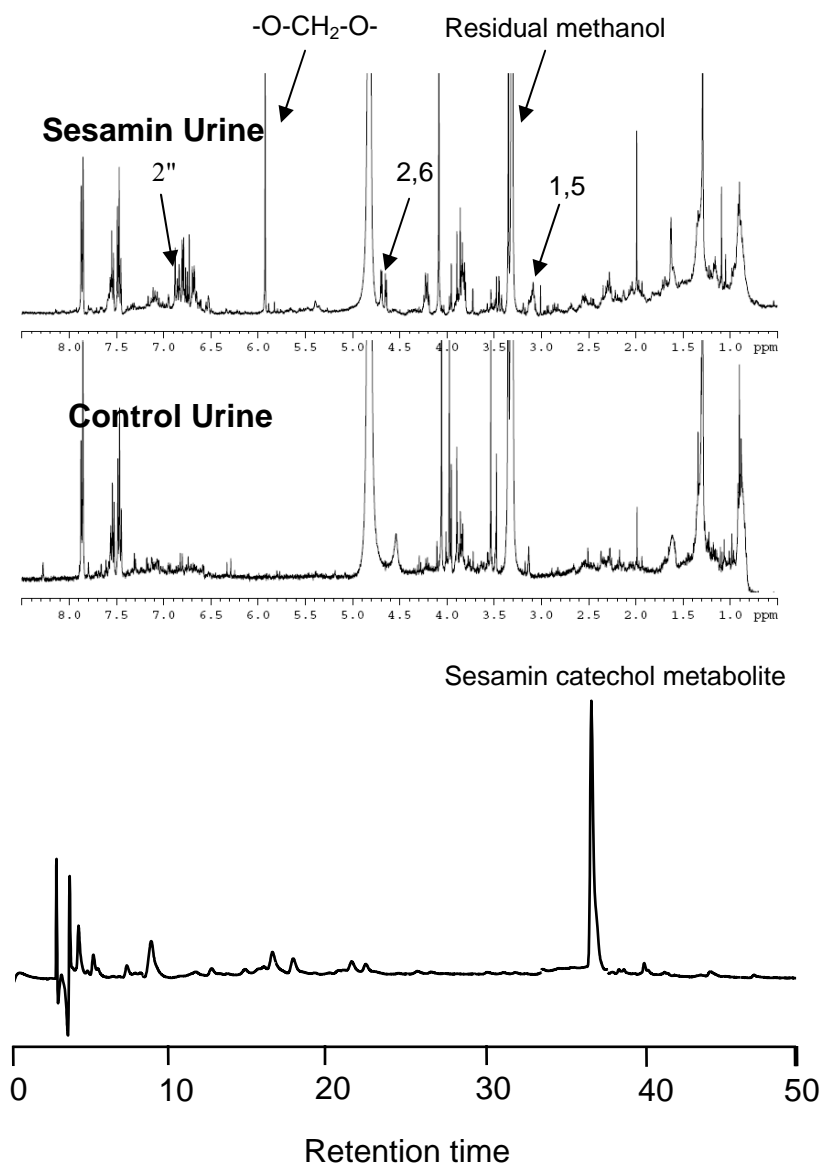


Figure 9. A) The comparison of  $^1\text{H-NMR}$  spectra of the chloroform extracts of control urine and that of treatment urine after ingestion of sesamin. Signals are marked to the corresponding position on the metabolite molecule. B) Typical RP-HPLC chromatogram of chloroform extract of deconjugated human urine.

### *Urinary excretion of sesamin catechol metabolite*

Human volunteers (n=6) took muffins containing sesame-oil and the urinary excretion of sesamin, the major sesame oil lignan, in the form of its catechol metabolite, which was isolated and characterized in this thesis, was investigated. **Table 6** presents the urinary excretion of sesamin catechol metabolite over four 12-hour periods as quantified by the NMR technique explained *vide supra*. The majority of the sesamin metabolite was excreted during the first two 12-hour periods. For three subjects, small amount of the metabolite was detected in the third period and no metabolite was detected in urine samples collected over the fourth period. The excretion of the sesamin catechol metabolite in urine ranged 22.2-38.6% (Mean $\pm$ SD 29.3 $\pm$ 5.6%) of ingested dose.

No specific peak was detected in HPLC chromatogram or NMR spectra, which could be assigned to a sesamol metabolite or other sesamin metabolites. Moreover, no sesamin or sesamol was detected by HPLC-DAD in urine samples, which is in agreement with Coulman *et al.* (2005). Preliminary experiments showed that sesamol is degraded under acidic condition of stomach. NMR data showed that the catechol metabolite possessing MDP signal ( $\delta$  5.91) in the H-NMR spectrum of urine extract does not contain any oxygen atom between its furofuran and benzene rings. This structural differentiation confirms the lack of contribution of sesamol, the second major lignan consumed in this study, to the production of the catechol metabolite.

Investigation of sesamin human metabolism in this thesis revealed that almost 30% of dietary sesamin is excreted in the form of a major catechol metabolite. It was already shown that formation of intermediate complexes with cytochrome is more extensive with MDP compounds that contain electron donating substituents on their aromatic ring while CO production is favored by electron withdrawing substituents (Yu & Wilkinson, 1980). Therefore, it is plausible that after the formation of the first catechol group on the sesame molecule, the metabolite may bind to CYP 450 and the metabolism of sesamin does not continue to further oxidative demethylation of the second methylenedioxy group on the molecule after the formation of a catechol group in the first phenyl ring.



Table 6. Urinary excretion of sesamin catechol metabolite (mg) during a 4×12-hour-periods after a single oral dose of sesamin (180 mg).

Period (hour)	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6
	Excreted amount of the metabolite (mg)					
0-12	39.0	15.8	49.6	15.1	54.2	38.7
12-24	12.5	50.3	1.1	25.2	ND	ND
24-36	0.4	1.1	ND	3.0	ND	ND
36-48	ND	ND	ND	ND	ND	ND
Total	51.9	67.2	50.8	43.3	54.2	38.7
	Excreted amount of the metabolite (Percent of ingested dose)					
Total	29.8	38.6	29.2	24.8	31.1	22.2

ND= Not Detected. The detection limit of the method for the metabolite was 0.5 µg/mL of urine using a signal-to-noise ratio of 3 and the limit of quantification was 2 µg/mL of urine using a signal-to-noise ratio of 10 on <sup>1</sup>H-NMR spectrum.

Apart from 29.3% sesamin, which is excreted in the form of its catechol metabolite, the fate of the rest of the dose ingested is not clear. It was previously shown that only about 0.1% of sesamin is absorbed through the lymph (Hirose *et al.*, 1991; Umeda-Sawada *et al.*, 1999) and that >40% of orally ingested sesamin was excreted as metabolites in the bile in rats (Nakai *et al.*, 2003). It has also been shown that sesamin can be converted to enterolactone and enterodiol in humans (Penalvo *et al.*, 2005; Coulman *et al.*, 2005). The total recovery of enterolactone and enterodiol, after four weeks ingestion of sesame seeds, was reported as 14.9% of total seed lignans consumed by humans (Coulman *et al.*, 2005). In this thesis, only small amounts of enterolactone were detected on the HPLC chromatograms after the single dose intervention with sesame oil. However, it was shown that the magnitude of plant lignans conversion to enterolactone and enterodiol is increased by longer intervention times (Smeds *et al.*, 2004), which may account for the low conversion of sesame oil lignans to enterolactone in this study. Moreover, Coulman *et al.* (2005) and Penalvo *et al.* (2005) used sesame seeds instead of sesame oil, which was used in this thesis.

Sesamin, like other lipophilic chemicals possessing MDP moiety, undergoes oxidative biotransformation and demethylation of its MDP group to form hydroxylated catechol metabolite (Murray, 2000). There is growing evidence that the epithelial cells (enterocytes) of human small intestine may provide the first site for cytochrome P450 (CYP)-catalyzed metabolism of orally ingested compounds (Scott Obach *et al.*, 2001). It has also been proposed that high luminal concentration of ingested compounds may lead to a saturation of intestinal wall metabolism (Glaeser *et al.*, 2004) and possibly change the absorption pattern. Moreover, initially, conjugation of phenolic compounds was thought to occur mainly in the liver, but phase II metabolism can also take place during uptake in the intestine (Jansen *et al.*, 2005). Therefore, the investigation of the content of sesamin, sesamin catechol metabolite or conjugated sesamin metabolite in portal vein after ingestion of sesamin can reveal the form of absorption and also the contribution of intestinal metabolism and conjugation on final excreted sesamin catechol metabolite. Moreover, study on the possible efflux of sesamin or its metabolite from enterocytes back to lumen by p-glycoproteins may provide more data for better understanding the amount of sesamin absorbed.

#### *Effect of acidic condition on sesaminol triglucoside*

Incubation (37°C for 1, 3 and 20 hours) of DSF extract or sesaminol triglucoside standard in acidic condition (pH=0.8-1.5, HCl) to simulate the acidic condition of stomach, did not cause any hydrolysis of sesaminol triglucoside as no peak corresponding to its sesaminol aglycone or other sesaminol glucosides were detected and incubation of sesaminol triglucoside standard did not generate any peak with ions corresponding to other lignan glucosides in its spectrum. However, the acidic incubation generated three new peaks beside sesaminol triglucoside, which possessed similar UV spectra and major ions to sesaminol triglucoside in their spectra in both positive [ $S_3G+Na$ ]<sup>+</sup>=879.2 and negative [ $S_3G-H$ ]<sup>-</sup>=855.2 ESI modes. Stereochemical changes of the bonds in position 2 and 6 of furofuran ring can possibly explain the three new chromatographic peaks since four different

stereochemical isomers of sesaminol were already reported (Nagata *et al.*, 1987). This reaction can possibly be stimulated in aqueous solution since sesaminol triglucoside is a water soluble compound.

### **Interactions between sesamin and phytosterols with regard to lipid metabolism (Paper VI)**

Sesamin (Hirose *et al.*, 1991) and phytosterols (de Jong *et al.*, 2003) are known to inhibit the incorporation of cholesterol into mixed micelles and, as a result, lower its absorption. Since sesamin and phytosterols competitively inhibit the absorption of other fat-soluble nutrients, it was investigated in a rat model if they may affect each other's physiological effects possibility by reducing their respective bioavailabilities. The phytosterols in the diet did not affect  $\gamma$ -tocopherol concentrations in plasma or liver. Sesamin, on the other hand, elevated  $\gamma$ -tocopherol in both tissues ( $p < 0.001$ ) in agreement with previously published data (Kamal-Eldin *et al.*, 1995, 2000). However the magnitude of this effect was reduced when the content of phytosterols in diet was increased ( $p < 0.01$ ).

Both sesamin and phytosterols individually decreased liver cholesterol to a small extent, albeit not significantly. However, when sesamin and phytosterols (1.1 g/kg diet) were fed together, a significant interaction was observed that nullified the individual cholesterol-lowering effects of the compounds ( $p < 0.05$ ). Similarly, sesamin intake increased the percentage of arachidonic acid (20:4n-6) and dihomo- $\gamma$ -linolenic acid (20:3n-6) in liver lipids ( $p < 0.05$ ) in agreement with Mizukuchi *et al.* (2003). Again, the effect of sesamin on dihomo- $\gamma$ -linolenic acid was abolished when the content of phytosterols was increased in the diet. Sesamin exerts its effects on arachidonic acid content both through reduction of its biosynthesis by inhibiting  $\Delta 5$ -desaturase (Shimizu *et al.*, 1991; Chavali & Forse, 1999) and possible reduction of its  $\omega$ -hydroxylation by inhibiting CYP4F2 (Powell *et al.*, 1998). Therefore, the interpretation of the effect of sesamin on arachidonic acid content is more complex being dependant on the inhibition of the activities of  $\Delta 5$ -desaturase, CYP4F2 and  $\beta$ -oxidation by sesamin.

The findings in this thesis suggest that dietary phytosterols may reduce the bioavailability and, hence, the biopotency of sesamin or its metabolites. Consumption of phytosterols was already shown to reduce the bioavailability of  $\beta$ -carotene and  $\alpha$ -tocopherol (Richelle *et al.*, 2004). The facts that both sesamin and phytosterols are constituents of vegetable oils and that sesamin inhibit the absorption of cholesterol make it possible that they compete for incorporation into mixed micelles, which is a necessary step for absorption. In addition, phytosterols are ligands for liver X receptors (LXRs), which can induce the expression of some p-glycoproteins (de Jong *et al.*, 2003) that may cause efflux of sesamin from enterocytes back to the lumen and reduce its absorption. It was recently shown that administration of sesamin and stanol can reduce the formation of enterodiol in LDL receptor-deficient mice (Penalvo *et al.*, 2006). Moreover, it was shown that almost 40% of sesamin is recovered in the form of its metabolites in the bile (Nakai *et al.*, 2003) showing the possible importance of first pass contribution (metabolism in intestinal cells and liver) in the metabolism of sesamin, which

eventually is converted to enterolactone and enterodiol by intestinal microflora. Therefore, any reduction in sesamin absorption may reduce the metabolites in the first pass, which may contribute to the reduction of final metabolic products (enterolactone and enterodiol) as was observed by Penalvo *et al.* (2006).

It was found that 0.1% of ingested sesamin is absorbed through the lymph in rats (Hirose *et al.*, 1991; Umeda-Sawada *et al.*, 1999). However, we have shown in this thesis that almost 30% of dietary sesamin is excreted in the form of a catechol metabolite by humans (**Paper V**). Investigation of the pharmacokinetics of this metabolite and its urinary excretion in the presence (single dose or long-term intervention) or absence of phytosterols can provide data for better understanding of possible interaction of sesamin and phytosterols during absorption.

## Summary and conclusions

The finding of this thesis can be summarized as follow:

- Two new lignan glucosides, sesaminol diglucoside and sesamolinal diglucoside, were isolated from sesame seeds and characterized.
- Lignan glucosides were analyzed by a validated method. It was found that sesaminol triglucoside is the major lignan glucoside in sesame seeds and that almost 32% of total lignans in sesame seeds are in glucosylated form.
- Data on variation of lignans and lignan glucosides in different sesame seeds and products indicated that sesame seed is as a rich source of lignans as flaxseed. However, the variations in the contents of lignan in sesame seeds, oil and products are high, which should be considered for their possible incorporation in functional foods.
- Sesame seeds from plant with non-dehiscent capsules contained higher amounts of sesaminol and sesamolinal and lower amounts of sesamin and sesamolinal than sesame seeds from plants with shattering capsules.
- The contents of oil-soluble lignans in sesame seeds were negatively correlated to lignan glucosides. There were positive correlations between oil-soluble sesamin and sesamolinal and between glucosylated sesaminol and sesamolinal.
- A new NMR technique was developed to quantify the urinary metabolite of sesamin in humans based on using methylenedioxyphenyl signal ( $\delta$  5.91) in  $^1\text{H}$ -NMR spectra of deconjugated urine extracts to measure the metabolite content.
- Almost 30% of a single oral dose of sesamin (180 mg) is excreted in the form of a major catechol metabolite in human urine and the majority of excretion occurred in the first 24-hour period.
- Simultaneous consumption of phytosterols and sesamin reduced the biopotency of sesamin in its lipid modulating effects in rats.

## Future research

Little is known about the biosynthesis of sesame lignan glucosides and their role in the plant, which is needed to be elucidated.

It was shown in this thesis that 30% of sesamin is excreted in the form of a catechol metabolite. However, little is known about sesamin absorption. Therefore the analysis of sesamin or its major metabolite(s) in portal vein can reveal the major form of absorption of sesamin and the possible contribution of intestinal metabolism in sesamin absorption. It was already known that just 0.1% of sesamin is absorbed through the lymph in rats. Nevertheless, all the pharmacokinetic data available is for sesamin and therefore, study on the pharmacokinetics of the sesamin catechol metabolite is necessary.

Comparison between pharmacokinetic data of sesamin or its metabolite in presence or absence of phytosterols can also be used for interpretation of the interaction between sesamin and phytosterols observed in this thesis.

The possible contribution of p-glycoproteins and cytochromes in absorption of sesamin and the possible interaction of sesamin or other sesame seed lignans with nuclear receptors especially pregnane X receptor (PXR), liver X receptor (LXR) and constitutive androstane receptor (CAR) can provide a better insight of the effects of sesame lignans on human metabolism.

In this thesis, major sesame lignan glucosides were isolated and quantified, however little is known about their metabolism, absorption and possible contribution in the production of mammalian lignans. Therefore, studies on the metabolism and absorption of lignan glucosides are necessary.

Negative correlations between the consumption of phytoestrogens and breast and prostate cancer have been shown. However, little is known about the contribution of sesame seed lignans to phytoestrogen intake, which is important for studies aiming at establishing such diet/health relations.

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