

# **The Phenolic Complex in Flaxseed**

**Analysis, structural features and bioactivity**

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## Abstract

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Flaxseed is the richest plant source of the lignan secoisolariciresinol diglucoside (SDG). In flaxseed, SDG exists in an oligomeric structure with 3-hydroxy-3-methyl glutaric acid (HMGA) forming a phenolic complex together with *p*-coumaric acid and ferulic acid glucosides and herbacetin diglucoside (HDG). Epidemiological and animal studies indicate protective effects of flaxseed and SDG towards hormone-dependent cancers and cardiovascular diseases, and reducing effect toward cholesterol levels in blood. Knowledge about the structural features and properties of the phenolic complex are required to further understand bioavailability, bioconversion and bioactivity of flaxseed lignans in humans and animals, the biosynthesis in flaxseed, as well as if it may affect technology and quality of food products containing flaxseed or the phenolic complex.

A new fast and simple high-performance liquid chromatographic (HPLC) method was developed for analysing secoisolariciresinol diglucoside (SDG), *p*-coumaric acid glucoside and ferulic acid glucoside, based on direct hydrolysis of defatted flaxseed flour using alkali. Variations in SDG, *p*-coumaric acid glucoside and ferulic acid glucoside content were reported in flaxseed samples and bread products containing flaxseed. The composition and properties of flaxseed phenolic complex were studied by reversed-phase liquid chromatography and gel filtration fractionation. Results indicate that the phenolic glucosides exist in oligomers with variable molecular sizes. A complicated linkage pattern and/or possibly interactions with other components may contribute to the observed complexity.

SDG and the phenolic complex showed similar hydrogen-donating abilities to ferulic acid but higher than  $\alpha$ -tocopherol in the DPPH inhibition method, suggesting that SDG was the only active antioxidant in the phenolic complex. Contradicting results were obtained on the effect of SDG on levels of Vitamin E and cholesterol in two rat studies.

*Keywords:* Phenolic complex, secoisolariciresinol, *p*-coumaric acid glucoside, ferulic acid glucoside, flaxseed, bread

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## Sammanfattning

Lin är en traditionell oljeväxt odlad sedan urminnes tider. Linfröet används i mat och djurfoder och ur fröet kan olja pressas, s.k. linolja, som används i t.ex. linoljefärg. I växtvärlden är linfrö en av de rikaste tillgångarna till fytoöstroget secosolariciresinol diglukosid (SDG) och omega-3 fettsyran linolensyra. Andra viktiga komponenter i linfrö är lösliga kostfibrer, som sänker kolesterol, och proteiner, som har emulsionsstabiliserande egenskaper jämförbara med gelatin. Epidemiologiska och djurstudier tyder på att linfrö och SDG har skyddande egenskaper mot hormon-relaterade cancerformer och hjärtkärlsjukdomar, och har kolesterolsänkande egenskaper. Idag tillsätts linfrö till många produkter med avsikt att berika livsmedlet med linolensyra. Dessutom säljs linfröextrakt innehållande det fenoliska komplexet av många företag. De höga halterna av cyanogena glykosider och kadmium samt en laxerande verkan begränsar dock intaget av linfrö och Livsmedelsverket avråder från en konsumtion av linfrö högre än 2 matskedar per dag.

I linfrö ingår SDG i raka symmetriska oligomerer tillsammans med glutarsyraderivat (HMGA) och i sin tur ingår dessa oligomerer i ett fenoliskt komplex tillsammans med andra fenoliska glukosider. I tarmen omvandlas SDG av mikroorganismer till enterolakton och enterodiol som tas upp i blodomloppet och genomgår enterohepatisk cirkulation för att sedan utsöndras med urinen. För att förstå upptag och metabolism av SDG måste vi veta mer om komplexets struktur och egenskaper. Strukturen är också viktig för att närmare förstå biosyntesen av SDG i växten.

I denna avhandling har en ny, snabb och enkel analysmetod utvecklats baserad på en direkt alkalisk hydrolysering av avfettat linfrömjöl. Med denna metod analyserades halten av SDG, *p*-kumarsyreglukosider och ferulasyreglukosider i olika linfröprover från två olika platser i Sverige. Halten av dessa fenoliska glukosider har också analyserats i bröd med linfrön. Det fenoliska komplexets sammansättning och egenskaper har studerats med kromatografiska metoder. Resultatet tyder på att de fenoliska glukosiderna ingår i en komplicerad struktur med bred molekylviktsfördelning. Ett komplicerat bindningsmönster eller interaktioner med andra okända strukturer kan bidra till komplexiteten. I en studie med radikalen DPPH hade SDG och det fenoliska komplexet liknande vätedonerande egenskaper som ferulasyra men högre än  $\alpha$ -tokoferol. Detta tyder på att SDG är den enda verkande antioxidanten i det fenoliska komplexet. Epidemiologiska studier indikerar att det finns ett samband mellan höga blodvärden av E vitamin och en minskad risk mot hjärtkärlsjukdomar och cancer. Därför har SDG's och det fenoliska komplexets påverkan på halten av E vitamin och kolesterol i plasma och lever studerats i två djurstudier på råttor. Intag av 0,1 % SDG sänkte halten E vitamin i råttplasma och lever och höjde kolesterolet i lever. I den andra studien med olika doser av SDG (0,1-0,0125 %) hade SDG ingen verkan på E vitamin eller kolesterol i råttor.

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## Appendix

The present thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I. Eliasson, C., Kamal-Eldin, A., Andersson, R. & Åman, P. 2003. High-performance liquid chromatographic analysis of secoisolariciresinol diglucoside and hydroxycinnamic acid glucosides in flaxseed by alkaline extraction. *Journal of Chromatography A* 1012(2), 151-159.
- II. Strandås, C., Kamal-Eldin, A., Andersson, R. & Åman, P. 2008. Phenolic glucosides in bread with flaxseed. *Food Chemistry*
- III. Strandås, C., Kamal-Eldin, A., Andersson, R. & Åman, P. 2008. Composition and properties of flaxseed phenolic oligomers. *Food Chemistry*
- IV. Frank, J., Eliasson, C., Leroy-Nivard, D., Budek, A., Lundh, T., Vessby, B., Åman, P. & Kamal-Eldin, A. 2004. Dietary secoisolariciresinol diglucoside and its oligomers with 3-hydroxy-3-methyl glutaric acid decrease vitamin E levels in rats. *British Journal of Nutrition* 92, 169-176.
- V. Strandås, C., Åman, P., Lundh, T. & Kamal-Eldin, A. 2008. No effect of increased intake of secoisolariciresinol diglucoside on tocopherol and cholesterol levels in rat plasma and liver. *Manuscript to be submitted*.

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### *Contribution of the author to the papers*

- I-III, V** Planned main part of experimental work, the statistical evaluation and wrote the manuscript together with the co-authors.
- IV** Performed part of the experimental work and took part in the writing of the manuscript.

## List of abbreviations

|             |                                                            |
|-------------|------------------------------------------------------------|
| Anhydroseco | anhydrosecoisolariciresinol                                |
| CEAD        | coulometric electrode array detection                      |
| CEHCs       | carboxyethyl hydroxichromans                               |
| CoA         | coenzyme A                                                 |
| CVD         | cardiovascular disease                                     |
| CYP450      | cytochrome P450                                            |
| CYP3A       | cytochrome P450 3A                                         |
| DFF         | defatted flaxseed flour                                    |
| DPPH        | 1,1-diphenyl-2-picrylhydrazyl                              |
| ED          | enterodiol                                                 |
| EL          | enterolactone                                              |
| ER          | estrogen receptor                                          |
| FeAG        | ferulic acid glucoside                                     |
| GC-MS       | gas chromatography-mass spectrometry                       |
| HDG         | herbacetin diglucoside                                     |
| HDL         | high-density lipoprotein                                   |
| HMG         | 3-hydroxy-3-methylglutaryl                                 |
| HMGA        | 3-hydroxy-3-methyl glutaric acid                           |
| HMR         | hydroxymatairesinol                                        |
| HPLC        | high-performance liquid chromatography                     |
| HPTLC       | high performance thin layer chromatography                 |
| IGF-1       | Insulin-like growth factor-I                               |
| JECFA       | joint FAO/WHO expert committee on food additives           |
| LC          | liquid chromatography                                      |
| LDL         | low-density lipoprotein                                    |
| LDLr        | low-density lipoprotein receptor                           |
| MALDI-TOF   | matrix assisted laser desorption ionisation-time of flight |
| Min         | multiple intestinal neoplasia                              |
| MTBE        | methyl <i>tertiary</i> -butyl ether                        |
| MS          | mass spectrometry                                          |
| NMR         | nuclear magnetic resonance                                 |
| PLR         | pinoresinol/lariciresinol reductase                        |
| PXR         | pregnane X receptor                                        |
| RP          | reversed-phase                                             |
| SDG         | secoisolariciresinol diglucoside                           |
| Seco        | secoisolariciresinol                                       |
| SPE         | solid-phase extraction                                     |
| $\alpha$ -T | $\alpha$ -tocopherol                                       |
| $\gamma$ -T | $\gamma$ -tocopherol                                       |
| TBARS       | thiobarbituric acid reactive substances                    |
| TC          | total cholesterol                                          |
| TG          | triglycerides                                              |
| THF         | tetrahydrofuran                                            |
| TRAMP       | transgenic adenocarcinoma mouse prostate                   |
| VEGF        | vascular endothelial growth factor                         |
| VLDL        | very low-density lipoprotein                               |
| WHO         | World Health Organization                                  |

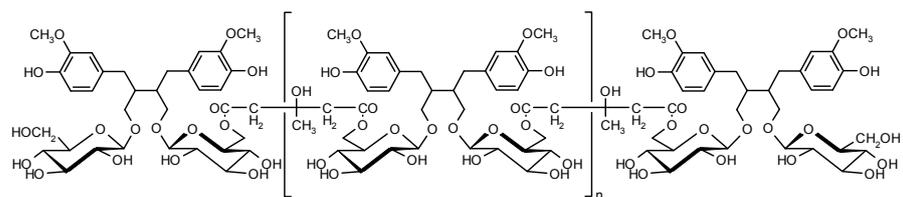
# Literature review

## Introduction

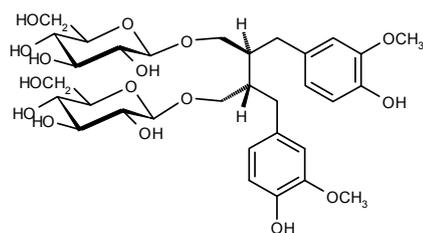
Flaxseed (*Linum usitatissimum* L.) is an ancient crop with a long history of cultivation. It was grown in the earliest agrarian societies in the Tigris and Euphrates valleys in Mesopotamia around 6000 B.C. (Oates, 1979). Traditionally, the oil from flaxseed (linseed oil) has mainly been used in industrial production of paints, paint thinners and linoleum, and the byproducts from oil production, such as pressed flaxseed cake, have been used in animal feed. Early references to flaxseed use as a food ingredient can be found in ancient Greek and Roman literature (Encyclopaedia Britannica). Today, whole or crushed flaxseed is included in bread or breakfast cereals as a healthy component but flaxseed is also consumed roasted in stews, porridge or drinks, e.g. in Ethiopia (Siegenthaler, 1994; Muir & Westcott, 2000). World production of flaxseed is currently led by Canada, China, India, the USA and the EU, with Canada the dominant producer (FAOSTAT, 2006).

Flaxseed is a small flat oval seed ranging in colour from yellow to brown (Freeman, 1995). The lignan secoisolariciresinol diglucoside (SDG) is widespread within the plant kingdom but the richest known plant source is flaxseed, where its level ranges from 0.5 to 1% (Figure 1) (Westcott & Muir, 1996b; Mazur & Adlercreutz, 1998; Johnsson *et al.*, 2000). Removal of the seed coat (hull) from flaxseed has proven difficult due to a layer of endosperm tissue adhering to the hull. The exact location of SDG in flaxseed has never been established (Wiesenborn, Tostenson & Kangas, 2003). A negative correlation has been found between SDG content and oil content in different fractions of dehulled flaxseed, indicating that SDG might be found in the hull (Madhusudhan *et al.*, 2000; Wiesenborn, Tostenson & Kangas, 2003). Other important nutrients from flaxseed are the flaxseed oil, which is rich in the omega-3 fatty acid  $\alpha$ -linolenic acid (39-44%); the water-soluble mucilage (2%), which contain a neutral and two acidic pectin-like polysaccharides (Warrand *et al.*, 2003); and albumin and globulin proteins (18-20%) (Oomah, Mazza & Kenaschuk, 1996).

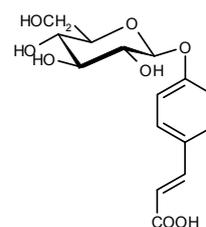
Limiting factors for flaxseed consumption are the high content of cyanogenic glycosides (100-300 mg hydrogen cyanide/kg seed) and cadmium (294-1543  $\mu\text{g}/\text{kg}$ ) (Rosling, 1993; Oomah *et al.*, 2007). In the absence of toxicological data, the provisional tolerable daily intake has been set to 12  $\mu\text{g}$  cyanide/kg body weight (WHO, 1996) and 1  $\mu\text{g}$  cadmium/kg of body weight (JECFA, 1993). With regard to the levels of cyanogenic glycosides, the intake of flaxseed should be limited to 10-20 g whole flaxseed per day and the National Food Administration in Sweden advises against usage of crushed or milled flaxseed, since the bioavailability of cyanides increases with disintegration. A high content of mucilage in flaxseed also restricts the daily intake to approximately 45 g due to a laxative effect in humans (Clark *et al.*, 2001). Thus, although flaxseed is the richest source of lignans, its dietary intake is limited.



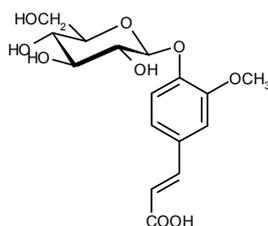
SDG-HMGA oligomers



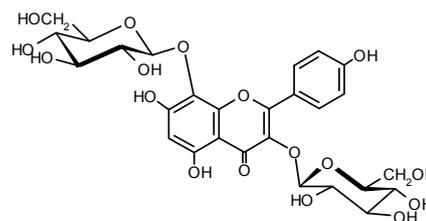
SDG



*p*-Coumaric acid glucoside



Ferulic acid glucoside



HDG

Figure 1. Structure of the phenolic glucosides in the flaxseed phenolic complex; secoisolariciresinol diglucoside 3-hydroxy-3-methyl glutaric acid oligomers (SDG-HMGA oligomers) (average  $n=3$ ), secoisolariciresinol diglucoside (SDG), *p*-coumaric acid glucoside, ferulic acid glucoside, and herbacetin diglucoside (HDG).

## The phenolic complex in flaxseed

The first article reporting a glucosidic phenolic complex being released from the flaxseed matrix by organic extraction using dioxane/ethanol was published by Klosterman & Smith (1954). Several years later, the phenolic complex obtained from flaxseed has been proposed to be composed of a structurally heterogeneous mixture of oligomers with SDG and 3-hydroxy-3-methyl glutaric acid (HMGA) together with other phenolic constituents (Figure 1) (Kamal-Eldin *et al.*, 2001; Ford *et al.*, 2001). Upon hydrolysis of the phenolic complex, phenolic compounds such as SDG and the hydroxycinnamic acid glucosides (*p*-coumaric acid glucoside and ferulic acid glucoside) are released (Johnsson *et al.*, 2002). Based on NMR

analyses, the phenolic complex has a linear and symmetrical structure with an average molecular weight of approx. 4000 Da and is composed of SDG and HMGA units covalently linked *via* ester bonds between the carboxylic carbon of HMGA and the C-6 of glucose residues in SDG, where HMGA is only present in the symmetrically esterified form (Kamal-Eldin *et al.*, 2001). The glucose residues of SDG are both ester-linked at C-6 of the glucose and some have been observed in the terminal position in the oligomers. The average ratio of terminal to intermediate SDG groups has been calculated as 1:4 and the SDG/HMGA ratio as 0.56/0.44. Other phenolic compounds found to be present in flaxseed include pinoresinol diglucoside (Qiu *et al.*, 1999), isolariciresinol (Meager *et al.*, 1999), matairesinol (Meager *et al.*, 1999; Liggins, Grimwood & Bingham, 2000) and ferulic, *p*-hydroxybenzoic, gentisic, vanillic and sinapic acids in free and/or bound forms (Kozłowska, Zadernowski & Sosulski, 1983; Dabrowski & Sosulski, 1984; Liggins, Grimwood & Bingham, 2000).

Recently, the hydroxycinnamic acid glucosides and the flavonoid herbacetin diglucoside (HDG) were discovered as components of the phenolic complex. The heterogeneity of the phenolic complex has been confirmed using matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) analysis of an aqueous ethanol extract from flaxseed hull (Struijs *et al.*, 2007). The authors reported that a complex spectrum was obtained, with clusters of peaks ranging from 2SDG+1HMGA to 5SDG+5HMGA. Preparative RP-HPLC fractionation of a partially hydrolysed flaxseed extract yielded HDG, HDG+HMGA and HDG+HMGA+SDG fragments, which were confirmed by MS/MS analysis. Moreover, *p*-coumaric acid glucoside was found to be ester-linked *via* its carboxyl group to the C-6 of glucose residues in SDG and not *via* HMGA as in HDG (Struijs *et al.*, 2008). A structural element of HMGA+SDG+ferulic acid glucoside was obtained using MS/MS but NMR analysis could not confirm any connection between SDG and ferulic acid glucoside. However, ferulic acid was found to be ester-linked to the C-2 position of glucose residues in SDG.

In the search for the biosynthetic pathway of SDG in flaxseed, Ford *et al.* (2001) applied radioisotopic incorporation of the SDG precursor L-[U-<sup>14</sup>C]-phenylalanine through different stages of seed development and discovered that SDG was accumulating at all stages, to reach its highest value at maturation. A mixture of different monomers and dimers of SDG-HMGA was obtained at the stage before seed maturation. Incorporation and formation of *p*-coumaric acid glucoside and ferulic acid glucoside occurred at an earlier stage of seed development. SDG is optically active with one dominant enantiomer in flaxseed, (+)-SDG of 99% (Ford *et al.*, 2001). The presence of two enantiomers of SDG in plants might suggest that there are two distinct biochemical pathways for SDG biosynthesis (Davin & Lewis, 2003). Biosynthesis of (+)-SDG *in planta* begins with an enantioselective dimerisation of two coniferyl alcohol units to produce (-)-pinoresinol by the protein pinoresinol synthase, which consists of a radical-forming oxidase and a 'dirigent protein'. (-)-Pinoresinol is then reduced by pinoresinol/lariciresinol reductase (PLR), *via* lariciresinol, to (+)-secoisolariciresinol (Seco) (Davin *et al.*, 1997; Davin & Lewis, 2003). This biosynthesis pathway of SDG may be true for

flaxseed, since PLR has been found in *Linum usitatissimum* L. (von Heimendahl *et al.*, 2005). During seed maturation, HMGA is attached to SDG mediated by coenzyme A (CoA)-activated HMGA (Ford *et al.*, 2001). In *Linum flavum*, the enzyme secoisolariciresinol dehydrogenase that converts secoisolariciresinol to matairesinol has been discovered (Xia *et al.*, 2000). In flaxseed, this conversion might take place to a minor extent since only a small amount of matairesinol has been detected (Meager *et al.*, 1999; Liggins, Grimwood & Bingham, 2000).

## **Principles of analysis of phenolic glucosides in flaxseed and bread**

### *Extraction and hydrolysis*

A critical step before extraction of phenolic compounds in foods is sample collection/storage and sample pre-treatment/clean-up (Tura & Robards, 2002). Drying and disintegration of samples by milling is important to obtain a homogeneous sample that facilitates the extraction of the phenolic compounds.

Flaxseed contains large amounts of non-polar lipids that must be removed before analysis of phenolic glucosides, most commonly by extracting milled flaxseed with non-polar solvents to obtain defatted flaxseed flour (DFF) (Table 1). The phenolic complex in DFF are extracted using more polar solvents such as dioxane/ethanol, aqueous ethanol or methanol in combination with heat and mixing (Johnsson *et al.*, 2000; Westcott & Muir, 1996a; Muir & Westcott, 2000). Recently, the phenolic complex were isolated from whole flaxseed by subcritical water extraction at high temperature in combination with high pressure (Cacace & Mazza, 2006). Subcritical water extraction decreases the dielectric constant of water and provides similar properties to ethanol or methanol. Recovery of SDG, *p*-coumaric acid glucoside and ferulic acid glucoside was 80% after subcritical water extractions at 140-160°C and 5.2 Pa. In another study, microwave-assisted extraction was used to quantify SDG, *p*-coumaric acid and ferulic acid glucosides in flaxseed and was found to shorten the time of extraction and hydrolysis of traditionally used methods (Beejmohun *et al.*, 2007). However those authors failed to refer to the correct yield of other methods and claimed that their method produced higher yield, but their content of SDG in flaxseed was within the range published by Johnsson *et al.* (2000).

Table 1. Summary of methods used for the quantification of lignans in flaxseed samples. Lignan content given as mg/g seed

| Lignan                 | Content               | Sample                                       | Extraction                                                                                            | Hydrolysis                                                                                                                      | Chromatography            | Reference                              |
|------------------------|-----------------------|----------------------------------------------|-------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------|---------------------------|----------------------------------------|
| SDG                    | 5.8-18.5 <sup>c</sup> | DFF <sup>a</sup> (0.5 g)<br><i>n</i> =10     | MeOH/water (7:3, v/v, 10 L,<br>3 h, 60 °C)                                                            | aq. <sup>b</sup> NaOH (0.1 M, 3 h, 20 °C)                                                                                       | RP-HPLC<br>UV 280 nm      | Westcott &<br>Muir, 1996b <sup>c</sup> |
|                        | 6.1-13                | DFF (0.5 g)<br><i>n</i> =29                  | Dioxane/EtOH (1:1, v/v, 10<br>mL, 16 h, 60 °C)                                                        | 1.aq. NaOH (0.3 M, 48 h, 20 °C)<br>2.SPE <sup>d</sup> C18                                                                       | RP-HPLC<br>UV 280 nm      | Johnsson <i>et al.</i> , 2000          |
|                        | 14                    | DFF (0.5 g) <i>n</i> =1                      | Dioxane/EtOH (1:1, v/v)                                                                               | NaOH <sup>c</sup> (0.1 M, 2 h, 40 °C)                                                                                           | HPTLC                     | Coran <i>et al.</i> , 2004             |
|                        | 10.1                  | Whole flaxseed<br>(2 g) <i>n</i> =1          | 1.Subcritical water extraction<br>(160 °C, 5.2 MPa).<br>2.EtOH/ THF <sup>f</sup> (1:1, v/v)           | 1.aq. NaOH (1 M, 1 h, 20 °C)<br>2.Precipitation with MeOH                                                                       | RP-HPLC<br>UV 280 nm      | Cacace &<br>Mazza, 2006                |
|                        | 9.8 <sup>g</sup>      | Pressed flaxseed<br>cake (0.5 g) <i>n</i> =1 | Microwave-assisted extraction in 20 mL MeOH/water (7:3, v/v)<br>and NaOH (0.1 M) (50-150 W; 1-15 min) |                                                                                                                                 | RP-HPLC<br>UV 280 nm      | Beejmohun<br><i>et al.</i> , 2007      |
| Anhydro-<br>seco, Seco | 4.2, 12.6             | Milled flaxseed<br>(0.5 mg) <i>n</i> =2      | n.a.                                                                                                  | 1.aq. HCl (5 mL, 1.5 M, 100 °C, 3 h)<br>2.EtOAc/ MTBE <sup>h</sup> (1:1, v/v; 3x2 mL)                                           | GC-MS                     | Liggins <i>et al.</i> ,<br>2000        |
|                        | 5.9                   | DFF (0.6 g) <i>n</i> =1                      | MeOH /water (7:3, v/v,<br>12 mL, 3 h, 60 °C)                                                          | 1.HCl <sup>e</sup> (2 M, 2.5 h, 100°C)<br>2.EtOAc (2 x 10 mL)                                                                   | RP-HPLC<br>UV 204nm       | Charlet <i>et al.</i> ,<br>2002        |
| Seco                   | 0.8                   | Milled flaxseed<br>(0.1 g) <i>n</i> =1       | Homogenisation in acetate<br>buffer                                                                   | 1.β-Glucuronidase <sup>b</sup> (37 °C, 1 day)<br>2.SPE C18                                                                      | RP-HPLC<br>UV 280 nm & MS | Obermeyer <i>et al.</i> , 1995         |
| Seco, ED,<br>EL        | 1.0-3.2<br>(μmol/g)   | Milled flaxseed<br>(0.5-1.0 g) <i>n</i> =10  | n.a.                                                                                                  | 1.Human faecal inoculum, 24 h<br>2.β-Glucuronidase <sup>b</sup> (37 °C, overnight)<br>3.SPE C18 & DEAE-Sephadex OH <sup>-</sup> | GC/MS                     | Thompson <i>et al.</i> , 1997          |

<sup>a</sup> Defatted flaxseed flour (DFF) <sup>b</sup> aq. aqueous solution <sup>c</sup> According to the patent by Westcott & Muir, 1996a. <sup>d</sup> Solid-Phase Extraction (SPE) <sup>e</sup> No information on aqueous or methanol solution <sup>f</sup> Tetrahydrofuran (THF) <sup>g</sup> Calculated value of SDG from an estimated oil content (40%) <sup>h</sup> Methyl *tertiary*-butyl ether (MTBE)

Alkaline hydrolysis in water or methanol is used to break ester-linkages to release SDG, *p*-coumaric acid glucoside and ferulic acid glucoside or their methyl esters from the phenolic complex (Johnsson *et al.*, 2000). Other methods using acid hydrolysis in combination with heat break ester and ether linkages to release glucose residues and obtain Seco (Figure 2) (Mazur *et al.*, 1996; Charlet *et al.*, 2002). Depending on the acid concentration, Seco is destabilised by dehydration to yield anhydrosecoisolariciresinol (Anhydroseco, also called Shonanin). Maximum content of Anhydroseco can be obtained in hot acid (2 M HCl, 100°C, 2.5 h) without any trace of Seco, and is relatively stable with minor degradation of 14% (Charlet *et al.*, 2002).

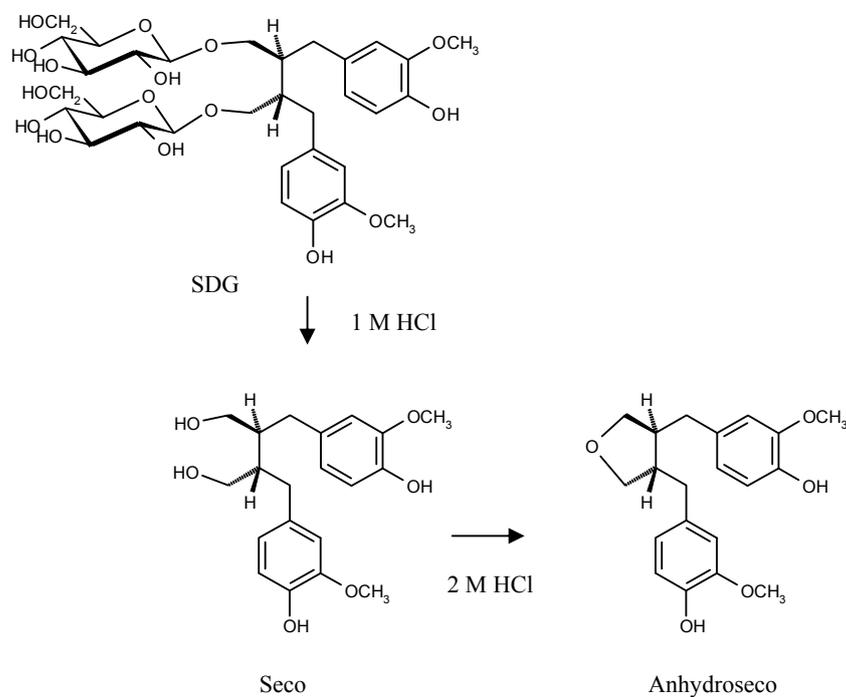


Figure 2. Transformation of secoisolariciresinol diglucoside (SDG) during acid hydrolysis to secoisolariciresinol (Seco), then anhydrosecoisolariciresinol (Anhydroseco).

Thompson *et al.* (1997) used *in vitro* fermentation of flaxseed with human faecal microbiota to mimic the human colonic environment and indirectly determine the content of SDG by the production of Seco, ED and EL.  $\beta$ -Glucuronidase is more commonly used in deconjugation of lignans in biological samples, but has also been used after faecal fermentation or directly on DFF to obtain Seco (Thompson *et al.*, 1997). Mazur *et al.* (1996) observed a low yield of SDG when  $\beta$ -glucuronidase from *Helix pomatia* was incubated with DFF, indicating that  $\beta$ -glucuronidase under these experimental conditions has low ability to hydrolyse lignan glucosides in DFF. In another study,  $\beta$ -glucuronidase from *H. pomatia* and  $\beta$ -glucosidase from almonds were able to hydrolyse isolated SDG to Seco by 90% and 13%, respectively (Milder *et al.*, 2004).

A summary of methods for quantification of lignans, SDG and Seco in freeze-dried flaxseed bread products after extraction followed by hydrolysis is presented in Table 2. Muir & Westcott (2000) developed a fast and simple method to quantify SDG that has been used by other authors to quantify SDG in flaxseed bread (Hyvärinen *et al.*, 2006; Pohjanheimo *et al.*, 2006). Thompson *et al.* (2006) and Milder *et al.* (2004) used complicated sample treatments with many work-up steps that might increase the risk of contamination or loss of lignans from the samples.

### *Chromatographic methods*

The most frequently used chromatographic method for quantification of SDG or the phenolic complex is reversed-phase high-performance liquid chromatography (RP-HPLC) with UV detection (Westcott & Muir, 1996a; Johnsson *et al.*, 2000). HPLC coupled to coulometric electrode array detection (HPLC-CEAD) is based on oxidation of lignans by applying electric potential on the analyte (Peñalvo & Nurmi, 2006). HPLC-CEAD is limited to the analysis of Seco, SDG, ED and EL, which have free phenolic hydroxyl groups ready to be oxidised. High performance thin layer chromatography (HPTLC) reduces the analysis time compared with HPLC by running several samples in a single run (Coran, Giannellini & Bambagiotti-Albert, 2004). Gas chromatography-mass spectrometry (GC-MS) is used for the analysis of Seco or ED and EL after derivatisation by silylation to increase the volatility and selectivity of the compounds (Thompson *et al.*, 1997; Peñalvo *et al.*, 2005; Thompson *et al.*, 2006).

In GC-MS, internal standards such as 5 $\alpha$ -androstan-3 $\beta$ , 17 $\beta$ -diol and stigmasterol have been used (Thompson *et al.*, 1997; Thompson *et al.*, 2006), but isotope-labelled lignans have also been synthesised and analysed together with food using isotope-dilution selective-ion-monitoring (Peñalvo *et al.*, 2005).

## Content of phenolic glucosides in flaxseed and bread

### *Flaxseed*

A summary of the quantification of SDG and the hydroxycinnamic acid derivatives in flaxseed is presented in Table 1. Variation in SDG content in different samples of flaxseed has been explored in a few articles (Westcott & Muir, 1996b; Johnsson *et al.*, 2000), while the variations in *p*-coumaric acid glucoside and ferulic acid glucoside are not known. In general, lignan content from flaxseed after alkaline and acid hydrolysis in the aforementioned studies ranged from 5.8-18.5 mg/g seed and 4.2-12.6 mg/g seed, respectively. The lowest yield of lignans was obtained by faecal fermentation with human inoculum (Thompson *et al.*, 1997). Factors affecting the content of lignans determined by different methods might be differences in degree of milling, time of extraction or hydrolysis and/or degradation, inefficient extraction and/or losses during work-up procedures. Flaxseed samples grown in three different locations in three different years were analysed for content of SDG by Westcott & Muir (1996b). The variation in the content of SDG was found to be primarily due to production year, the second most important factor being flaxseed variety and the third the growing site.

### *Bread*

Knowledge of the stability of SDG in different types of bread containing flaxseed or isolated SDG is limited. A short fermentation time (1 h 40 minutes) of whole grain wheat buns and sourdough rye bread fortified with SDG followed by baking at 225-250°C for 15-25 minutes has been found to have minor effects on the SDG content (Hyvärinen *et al.*, 2006). In another study, long fermentation time (24 h) with rye sourdough had negligible effects on rye lignans (Liukkonen *et al.*, 2003). The recovery of SDG from bread enriched with SDG can be 99.5%, indicating that SDG is unaffected by the baking process (Muir & Westcott, 2000). When flaxseed was incorporated into bread in another study, the recovery of SDG was 73-82% (Muir & Westcott, 2000; Milder *et al.*, 2004). Hall *et al.* (2005) observed an increased recovery of SDG, from 40 to 80%, after treating flaxseed-containing pasta with papain before extraction of the phenolic complex from the pasta matrix. Those authors suggested that SDG is entrapped by the gluten network in pasta. A summary of lignan content in commercial bread containing flaxseed is presented in Table 2. After alkaline hydrolysis, the content of SDG in flaxseed bread ranges from 4.1-136 mg/100 g fresh weight (Muir & Westcott, 2000). After faecal fermentation, the content of Seco, ED and EL varies from 5.3-32.4 µmol/100 g fresh weight. The lower yield obtained by Milder *et al.* (2005) and Nesbitt & Thompson (1997) might be due to inefficient extraction and/or losses of lignans during the work-up procedures.

*In vitro* fermentation with faecal inoculum of home-made bread containing variable amounts of ground flaxseed of the variety Linott indicated a strong correlation between the percentage of flaxseed and the content of Seco, ED & EL in the fermented residues (Nesbitt & Thompson, 1997). A weaker correlation was found in commercial breads containing flaxseed of different varieties.

Table 2. Summary of methods for quantification of lignans in freeze-dried flaxseed breads. Lignan contents are given as mg/100 g seed

| Lignans       | Content                           | Flaxseed content     | Sample weight | Extraction                                               | Hydrolysis                                                                                                         | Chromatography       | Reference                        |
|---------------|-----------------------------------|----------------------|---------------|----------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------|----------------------|----------------------------------|
| SDG           | 4.1-136;<br><i>n</i> =10          | n.s. <sup>a</sup>    | 4 g           | MeOH/water<br>(7:3, v/v, 20 mL, 3 h, 60 °C)              | aq. <sup>b</sup> NaOH (0.1 M, 3 h, 20 °C)                                                                          | RP-HPLC<br>UV 280 nm | Muir & Westcott, 2000            |
|               | 20; <i>n</i> =1                   | 10% DFF <sup>c</sup> | 2.5 g         | MeOH/water<br>(7:3, v/v, 20 mL, 3 h, 60 °C)              | aq. NaOH (0.1 M, 3 h, 20 °C)                                                                                       | RP-HPLC<br>UV 280 nm | Hyvärinen <i>et al.</i> , 2006   |
|               | 63.3; <i>n</i> =1                 | 7.1% <sup>d</sup>    | 4 g           | MeOH/water<br>(7:3, v/v, 20 mL, 3 h, 60 °C)              | aq. NaOH (0.1 M, 3 h, 20 °C)                                                                                       | RP-HPLC<br>UV 280 nm | Pohjanheimo <i>et al.</i> , 2006 |
| Seco          | 11.9; <i>n</i> =1                 | n.s.                 | 1 g           | MeOH/water<br>(7:3, v/v; 0.3 M NaOH) (24 mL, 1 h, 60 °C) | 1. <i>H. pomatia</i> β-glucuronidase in acetate buffer (37 °C, overnight)<br>2.Diethyl ether extraction            | LC-MS/ MS            | Milder <i>et al.</i> , 2005      |
|               | 16, 24; <i>n</i> =2               | n.s.                 | 0.25-2 g      | MeOH/water<br>(7:3, v/v, 25 mL, 2 h, 60-70 °C)           | 1.aq. NaOH (0.1 M, 3 h, 20 °C)<br>2.SPE C18<br>3.β-Glucuronidase (37 °C, overnight)<br>4.SPE C18                   | GC-MS                | Thompson <i>et al.</i> , 2006    |
| Seco, ED & EL | 5.3-32.4 μmol/100 g; <i>n</i> =12 | 0.1-10.1%            | 0.5-1.0 g     | n.a.                                                     | 1.Human faecal inoculum, 24 h<br>2.β-Glucuronidase (37 °C, overnight)<br>3.SPE C18 & DEAE-Sephadex OH <sup>-</sup> | GC/MS                | Nesbitt & Thompson, 1997         |

<sup>a</sup>n.s. not stated <sup>b</sup>aq. aqueous solution <sup>c</sup> whole grain wheat was replaced with 10% defatted flaxseed flour (DFF) in the dough <sup>d</sup>whole & crushed flaxseed



To date, absorption of the hydroxycinnamic acid glucosides of flaxseed are unknown. However, it is generally known that bound hydroxycinnamic acid glucosides are metabolised to free phenolic acids by pancreatic and microbiotic esterases in the intestine (Kroon *et al.*, 1997; Andreassen *et al.*, 2001). In the liver and/or the intestinal mucosa, the hydroxycinnamic acids are conjugated with glucuronic acid and sulphuric acid to be excreted in the plasma, and in the bile for enterohepatic circulation (Mateos, Goya & Bravo, 2006), before being finally excreted in urine and faeces (Jacobson *et al.*, 1983).

#### *Absorption and metabolism of SDG in the intestine*

A significant reduction in urinary ED and EL and an increased ED/EL-ratio have been observed in humans treated with antibiotics (Setchell *et al.*, 1981), which highlights the importance of the microbiota in the colon for the bioavailability of the mammalian lignans. The absorption of ED and EL in the large intestine is affected by a whole range of different factors such as interindividual variation in the microbiota, intestinal transit time, structure of the lignans, composition of the diet and food matrix (Axelson *et al.*, 1982; Adlercreutz *et al.*, 1987; Adam *et al.*, 2002; Saarinen *et al.*, 2002; Smeds *et al.*, 2004). The stereochemical structure of SDG and Seco has been shown to determine the chirality and the composition pattern of ED and EL and their oxidation products (Saarinen *et al.*, 2002; Smeds *et al.*, 2004). Hydrolysis of *O*-glycosides into aglycones is one of the rate-limiting steps in the conversion of plant lignans to mammalian lignans (Saarinen *et al.*, 2002). Compartmentalisation and composition of the food and/or the flaxseed might determine the bioavailability of lignans. Crushing or grinding of whole flaxseed has been shown to increase the levels of plasma ED and EL in humans compared with whole flaxseed (Kuijsten *et al.*, 2005a). In *in vitro* fermentation models, the formation of ED and EL is increased by high amounts of carbohydrates (Cassidy, Hanley & Lamuela-Raventos, 2000), dietary fibre (Rowland *et al.*, 1999) and xylanase treated rye bran (Aura *et al.*, 2005). An increase in fat content in the diet decreases the urinary excretion of lignans in both rats and humans (Hallmans *et al.*, 1999).

After absorption of ED and EL, the lignans are conjugated with glucuronic acid and sulphuric acid by hepatic phase II enzymes (*e.g.* UDP glucuronosyltransferases and sulphotransferases) (Morton *et al.*, 1994; Adlercreutz *et al.*, 1995). The fate of the minor amount of Seco absorbed from the intestine is not known. About 50-60% of endogenous estrogen-conjugates in humans enters the enterohepatic circulation by excretion in the duodenum with the bile, followed by deconjugation with intestinal bacterial  $\beta$ -glucuronidase and sulphatase, and are reabsorbed from the intestine similarly to bile acids (Eriksson & Gustafsson, 1971; Adlercreutz & Martin, 1980). Conjugated ED and EL are subjected to the same enterohepatic circulation as estrogen (Axelson & Setchell, 1981; Adlercreutz *et al.*, 1987; Bach Knudsen *et al.*, 2003). Conjugated mammalian lignans are transported from the plasma to different tissues (*e.g.* uterus and kidneys) and are excreted in urine (Axelson & Setchell, 1981; Rickard & Thompson, 1998).

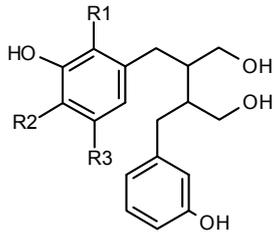
### *Phase I metabolism of SDG in liver*

Oxidation products of the mammalian lignans have been discovered both *in vitro* and *in vivo*, which suggests that they are substrates for cytochrome P450-mediated hydroxylation reactions in the liver (Figure 5). *In vitro*, up to 12 aliphatic and aromatic oxidation products of ED and EL have been found in rat hepatic aroclor-induced microsomes (Jacobs & Metzler, 1999). Most of the metabolites of ED and EL were also found in rat, pig and human uninduced microsomes. *In vivo*, minor amounts of aromatic oxidation products (<5%) were detected in human urine after flaxseed intake (Niemeyer & Metzler, 2002). Human pregnane X receptor (PXR), which is involved in the metabolism of CYP3A substrates in liver and intestinal tissues, was moderately activated *in vitro* by EL (Jacobs, Nolan & Hood, 2005). Furthermore, Seco might also be a substrate for phase I metabolism since oxidation products of Seco were discovered *in vitro* in aroclor-induced rat microsomes but these products have not been detected *in vivo* so far (Niemeyer & Metzler, 2002).

### *Pharmacokinetics of SDG*

Pharmacokinetic studies of ED and EL in humans performed after consumption of a single dose of SDG (1.31  $\mu\text{mol/kg}$  b.w.) showed peak plasma concentrations of ED after 15 h and EL after 20 h and mean elimination half-life of 4.4 h for ED and 12.6 h for EL (Kuijsten *et al.*, 2005b). The urinary excretion was 40% of the ingested dose of SDG, with the majority excreted as EL (58%). A positive correlation was obtained between the plasma ED and EL and the urine ED and EL in a two-month intervention study with premenopausal women (n=19) consuming 20 g flaxseed (Knust *et al.*, 2006).

Supplying single and multiple (10 days) doses of  $^3\text{H}$ -SDG (1.5 mg/d) to female rats for 48 h gave faecal lignan excretion of >50% of dose, urine lignan levels of 28-32% and plasma lignan excretion of 0.4% (equivalent to 1  $\mu\text{mol/L}$ ) (Rickard & Thompson, 1998). In that study, the plasma concentrations of ED, EL and Seco were higher than peak rat estrogen levels (300 pM) obtained by Butcher, Collins & Fugo (1974). Active sites of lignan metabolism, such as liver, kidney and uterus, had higher radioactivity compared with other non-gastrointestinal tissues, and multiple treatment enhanced the radioactivity in the liver by 50-80% compared with single treatment. In another study by Rickard & Thompson (2000a), giving single and multiple (10 days) doses of  $^3\text{H}$ -SDG to rats for 48 h resulted in 74-80% of urine excretion as EL, ED and Seco, and the urinary composition of lignans did not differ between treatment groups. The proportion of ED, EL and Seco present in the urine of the rats was 55, 10 and 13%, respectively, 24 h after ingestion. Seco has also been detected in human urine (Bannwart *et al.*, 1989).

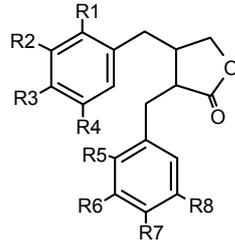


ED 2-hydroxy-ED

R1=H R1=OH  
R2=H R2=H  
R3=H R3=H

4-hydroxy-ED 6-hydroxy-ED

R1=H R1=OH  
R2=OH R2=H  
R3=H R3=OH

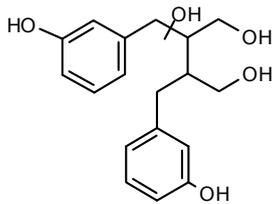


EL 2-hydroxy-EL 4-hydroxy-EL 6-hydroxy-EL

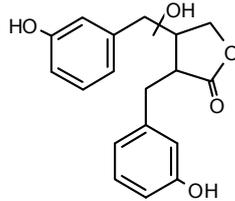
R1=H R1=H R1=H R1=H  
R2=OH R2=OH R2=OH R2=OH  
R3-7=H R3-4=H R3-6=H R3-4=H  
R8=OH R5=OH R7=OH R5=OH  
R6=OH R8=OH R6-7=H  
R7-8=H R8=OH

2'-hydroxy-EL 4'-hydroxy-EL 6'-hydroxy-EL

R1=OH R1=H R1=OH  
R2=OH R2=OH R2-3=H  
R3-7=H R3=OH R4=OH  
R8=OH R4-7=H R5-7=H  
R8=OH R8=OH



Aliphatic hydroxylation  
products of ED (4 products)



Aliphatic hydroxylation  
products of EL (6 products)

Figure 4. Aromatic and aliphatic oxidation products of enterodiol (ED) and enterolactone (EL) in rat, pig and human hepatic microsomes (Jacobs & Metzler, 1999).

## Health effects of flaxseed lignans

### *Hormone-dependent cancer*

#### Breast cancer

Epidemiological studies report different results regarding the protective effect of flaxseed lignans against hormone-dependent cancer forms (reviewed by Lof & Weiderpass, 2006). In an intervention study of newly diagnosed postmenopausal breast cancer patients ( $n=19$ ), a daily intake of flaxseed for a month reduced the expression of tumour cell proliferation markers, the protein Ki-67 and the epidermal growth factor receptor c-erbB2 and increased apoptosis, indicating protective effects of flaxseed lignans (Thompson *et al.*, 2005).

Animal studies with experimental cancer also indicate a protective effect of flaxseed and its components against breast cancer. The limitations and advantages of experimental animal models for human breast cancer were reviewed recently by Saarinen *et al.* (2007). For example, flaxseed given to carcinogen-treated rats or mice reduced tumour occurrence and size at the initiation and promotion stages of carcinogenesis (Serraino & Thompson, 1992b), tumour size at the late progress stage of carcinogenesis (Thompson *et al.*, 1996b), tumour growth and metastasis at the late progress stage of estrogen receptor (ER) negative carcinogenesis (Dabrosin *et al.*, 2002), and distant metastasis after surgical excision of established estrogen receptor negative human breast cancer tumour in nude mice, but did not prevent recurrence of cancer (Chen, Wang & Thompson, 2006).

The beneficial effects of flaxseed on breast cancer have partially been attributed to SDG. For example, carcinogenic-treated rats given SDG showed a reduction in tumour occurrence and size at the early promotion stage (Thompson *et al.*, 1996a), tumour multiplicity at the early promotion stage (Rickard *et al.*, 1999), tumour size and multiplicity at the late progress stage of carcinogenesis (Thompson *et al.*, 1996b), pulmonary metastasis (Li *et al.*, 1999), and distant metastasis after surgical excision of established ER negative human breast cancer tumour in nude mice, but not recurrence (Chen, Wang & Thompson, 2006). Intake of lignans before puberty might reduce the risk of breast cancer. Exposure to flaxseed or SDG during suckling of rat female offspring reduced tumour occurrence, size and number after ER negative carcinogen-treatment later in life (Chen *et al.*, 2003a). *In vitro*, ED and EL dose-dependently reduced ER negative cancer cell adhesion, invasion and migration steps involved in metastasis.

When SDG or flaxseed was combined with tamoxifen, a compound clinically used in the treatment of breast cancer, stronger inhibition of tumour growth was obtained (Chen & Thompson, 2003b; Chen *et al.*, 2007b). Combined treatment with tamoxifen and flaxseed reduced the expression of the progesterone receptor and insulin-like growth factor-I (IGF-1) and increased the expression of ER $\alpha$ . Furthermore, a synergistic protective effect was observed when SDG and flaxseed oil were administered together, indicating that SDG is not the only cancer-protective component in flaxseed (Chen, Wang & Thompson, 2006).

### Prostate cancer

Studies have been carried out regarding the protective effect of flaxseed against prostate cancer, with the suggestion of SDG as the protective component. Prostate tumour proliferation and the prostate-specific antigen were reduced in patients with prostate cancer given flaxseed in a low-fat diet (Demark-Wahnefried *et al.*, 2001; Demark-Wahnefried *et al.*, 2004), together with suppressed total and free testosterone levels (Demark-Wahnefried *et al.*, 2001). In the transgenic adenocarcinoma mouse prostate (TRAMP) model, flaxseed in a low-fat diet reduced prostate tumour size and proliferation marker Ki-67 and increased apoptosis (Lin, Switzer & Demark-Wahnefried, 2002). Flaxseed also inhibited prostate cell proliferation in rats with experimental cancer (Tou, Chen & Thompson, 1999). *In vitro* growth of the prostate cancer cell lines LNCaP, PC-3 and DU-145 was reduced by enterolactone and enterodiols and the androgen-sensitive cell line LNCaP was most affected, suggesting that these mammalian lignans contribute to the protective effect of flaxseed (Lin, Switzer & Demark-Wahnefried, 2001).

### Colorectal cancer

To date, no human studies have been performed on the effects of flaxseed or the phenolic complex on colorectal carcinogenesis and the existing animal studies are limited. A reduction in early markers of colon cancer risk (aberrant crypt and aberrant crypt foci) was observed in rats with experimental cancer given flaxseed or SDG in the short term (Serraino & Thompson, 1992a) and long term (Jenab & Thompson, 1996). In contrast, no effect of flaxseed on intestinal carcinogenesis was obtained in a multiple intestinal neoplasia (Min) mice model (van Kranen *et al.*, 2003; Oikarinen *et al.*, 2005), or when (-)-Seco was ingested by these mice (Pajari *et al.*, 2006).

### Possible mechanisms

The mechanism(s) involved in the protective effect of flaxseed and its lignans on hormone-dependent cancer forms are still very unclear. The main mechanism has been suggested to be related to lignans' ability to compete with estrogen for the estrogen receptor (ER) but other factors may also play a role (Saarinen *et al.*, 2007; Adlercreutz 2007). Diet-gene interaction between mammalian lignans and hormone-dependent cancer may modify the risk of cancer, *e.g.* premenopausal women expressing at least one allele for the gene cytochrome P450c17 $\alpha$  have a reduced risk of breast cancer (McCann *et al.*, 2002).

The estrogen receptor  $\alpha$  (ER $\alpha$ ) is highly expressed in uterus, testis, pituitary, ovary, epididymis and adrenal, whereas ER $\beta$  is expressed particularly in brain, kidney, prostate, ovary, lung, bladder, intestine and epididymis (Kuiper *et al.*, 1996; Enmark & Gustafsson 1999). Estrogenic activity of flaxseed has been discovered in rats by a dose-dependent lengthening of the estrous cycle (Orcheson *et al.*, 1998). A positive correlation between urinary lignan excretion and changes in the ratio of the estrogen bioactive metabolite 2-hydroxyestrone to 16 $\alpha$ -hydroxyestrone was observed in postmenopausal women, indicating that flaxseed

lignans have an influence on the metabolism of estrogens (Brooks *et al.*, 2004). Mammalian lignans bind weakly to rat uterine cytosol (Adlercreutz *et al.*, 1987), and *in vitro* they show low affinity for ER $\alpha$  and ER $\beta$  to act as partial agonists/antagonists of estrogens, also called estrogenic and antiestrogenic activity (Mueller *et al.*, 2004). EL has been shown to have estrogenic activity both *in vivo* in transgenic mice and at physiological concentrations *in vitro* by activating ER-negative mediated transcription with preference for ER $\alpha$  (Penttinen *et al.*, 2007).

The preventive effect on hormone-dependent cancer might differ with the ER status of the tumour. A tendency towards a lower risk of ER $\alpha$ -negative breast cancer was found with higher EL levels in postmenopausal women (Olsen *et al.*, 2004). In that prospective study, the levels of EL was not related to ER $\alpha$ -positive breast cancers. Both ED and EL interfere with testosterone at the binding sites of sex hormone binding globulin *in vitro* (Schöttner, Spitteller & Gansser, 1998) and EL is positively correlated with sex hormone binding globulin in human plasma (Zeleniuch-Jacquotte *et al.*, 2004). *In vitro*, EL moderately inhibits the human placental estrogen synthetase (aromatase) (Adlercreutz *et al.*, 1993) and aromatase in human preadipocytes (Wang *et al.*, 1994) but it is questionable whether the same effect is possible *in vivo* (Saarinen *et al.*, 2007). Other signalling pathways of ER may be effected by ED and EL (Saarinen *et al.*, 2007). For example in rats with experimental cancer, administration of flaxseed reduced the plasma IGF-I at pre-initiation and early promotion stages of carcinogenesis and a negative correlation was found between urinary excretion of ED, EL and Seco and plasma IGF-I (Rickard, Yuan & Thompson, 2000b). Flaxseed given to nude mice reduced vascular endothelial growth factor (VEGF), a key factor in promotion of tumour angiogenesis (Dabrosin *et al.*, 2002). Suppression of tumour growth in human colon cancer both *in vivo* in mice and *in vitro* by EL is suggested to be the result of apoptosis (Danbara *et al.*, 2005). In another *in vitro* study, EL suppressed growth by inducing apoptosis in human prostate cancer cells (Chen *et al.*, 2007a).

### *Cardiovascular disease*

Cardiovascular disease (CVD), the most common health problem in the world, is related to the major risk factors diabetes, hypertension, tobacco smoking, overweight, hypercholesterolaemia, physical inactivity and genetic factors (Kannel & McGee, 1979; WHO, 2002; Talmud, 2007). In a 12 year-prospective cohort study of Finnish men, those men with the highest serum EL levels had lower risk to die from coronary heart disease or CVD than those men with the lowest levels of EL (Vanharanta *et al.*, 2003). SDG is suggested to protect against CVD by its antioxidant activity and lowering effect on cholesterol (Prasad, 1999; Lucas *et al.*, 2004). In experimental rabbit studies with elevated cholesterol levels, the phenolic complex or SDG reduced the incidence of atherosclerotic lesions and plaques in the rabbit aorta (Prasad, 1999, 2005). However, no effect on the endothelial functions related to CVD risk factor was obtained in a human intervention study with normocholesterolaemic postmenopausal women administered the phenolic complex for six weeks (Hallund *et al.*, 2006b). Hypocholesterolaemic activity of SDG does not play a major role in slowing the progression of atherosclerosis (Prasad, 2007). A reduced progression of atherosclerosis by SDG in rabbits on a

regular diet following a high cholesterol diet may not have been due to lowering of serum lipids but possibly to a reduction in oxidative stress (Prasad, 2007).

The effects of flaxseed or the phenolic complex on plasma cholesterol, low-density lipoproteins (LDL) and triglycerides in human intervention studies and animal studies is presented in Table 3. In humans with hypercholesterolaemia, a daily intake of the phenolic complex reduced plasma cholesterol levels and LDL by 22 and 22%, respectively (Zhang *et al.*, 2007). However, in healthy postmenopausal women given almost the same dose of the phenolic complex from flaxseed there were no effects on plasma cholesterol, LDL, high-density lipoprotein (HDL) or triglycerides (Hallund *et al.*, 2006a). In long-term studies, plasma cholesterol and LDL were not reduced by flaxseed in men and women with the autoimmune disease *lupus nephritis* (Clark *et al.*, 2001) and in another study with postmenopausal women, a minor effect on plasma cholesterol was shown (Dodin *et al.*, 2008). In all experimental animal studies, flaxseed or SDG reduced the levels of plasma cholesterol except in an earlier study by Prasad (1997), in which plasma cholesterol levels increased in rabbit after consumption of flaxseed. Apo B-1 transgenic male and female mice with lipid profiles resembling humans were given ground flaxseed (20%) in a high cholesterol (0.1%) diet for 2 months with no effect on the expression of the following genes: low-density lipoprotein receptor (LDLr), 3-hydroxy-3-methylglutaryl (HMG) CoA reductase, phospholipid transfer protein, cholesterol 7 $\alpha$  hydroxylase, fatty acid synthase and acyl CoA oxidase (Pellizzon *et al.*, 2007).

One hypothesis is that flaxseed may protect against CVD through its estrogenic activity. Estrogen has been suggested to be protective towards cardiovascular disease through its beneficial effects on plasma lipoproteins, antiproliferation and vasodilation on the vasculature (reviewed by Farhat, Lavigne & Ramwell, 1996). Loss of estrogens associated with menopause in women has been suggested to increase the risk of cardiovascular disease (Mendelsohn, 2002). However, treatment with estrogen has not reduced the incidence of cardiovascular disease in postmenopausal women (Hulley *et al.*, 1998).

Table 3. Effects on plasma total cholesterol (TC), low-density lipoproteins (LDL), high-density lipoprotein (HDL) and triglycerides (TG) in animal studies and interventional studies with humans given whole, ground, milled and defatted flaxseed or the phenolic complex

| Model                                   | Diet and duration                                                        | Effects                                           | Reference                      |
|-----------------------------------------|--------------------------------------------------------------------------|---------------------------------------------------|--------------------------------|
| <b>Humans</b>                           |                                                                          |                                                   |                                |
| Men & women <sup>a</sup> (n=29)         | Partially defatted flaxseed (50 g/d) in muffins, 3 weeks                 | ▼ TC (5%), LDL (8%)                               | Jenkins <i>et al.</i> , 1999   |
| Women (n=20) <sup>b</sup>               | Ground flaxseed (40 g/d), 3 months                                       | ▼ TC (6%)                                         | Lucas <i>et al.</i> , 2002     |
| Women (n=85) <sup>b</sup>               | Ground flaxseed (40g/d) in bread for 1 year                              | ▼ TC (2%), HDL (1%)                               | Dodin <i>et al.</i> , 2008     |
| Women (n=25) <sup>b</sup>               | Ground flaxseed (40g/d) in bread, 3 months                               | n.e. <sup>c</sup>                                 | Lemay <i>et al.</i> , 2002     |
| Men & women <sup>d</sup> (n=8)          | Ground flaxseed (30 g/d) in sachets, 1 month                             | ▼ TC (11%), LDL (12%)                             | Clark <i>et al.</i> , 1995     |
| Men & women <sup>d</sup> (n=9)          | Ground flaxseed (30 g/d) in sachets, 1 year                              | n.e.                                              | Clark <i>et al.</i> , 2001     |
| Women (n=10)                            | Milled flaxseed (25 g/d) in muffins, 1 month                             | n.e.                                              | Cunnane <i>et al.</i> , 1995   |
| Women (n=16) <sup>b</sup>               | Whole flaxseed (25 g/d) in bars, 1 month                                 | n.e.                                              | Coulman <i>et al.</i> , 2002   |
| Men & women (n=38) <sup>a</sup>         | Phenolic complex (0.6 g SDG/d) in tablets                                | ▼ TC (24%), LDL (22%)                             | Zhang <i>et al.</i> , 2007     |
| Women (n=22) <sup>b</sup>               | Phenolic complex (0.5 g SDG/d) in muffins, 1.5 months                    | n.e.                                              | Hallund <i>et al.</i> , 2006a  |
| <b>Animals</b>                          |                                                                          |                                                   |                                |
| Rabbits <sup>e</sup> (n=5)              | Flaxseed (7.5 g/kg b.w. & d), cholesterol (1%), 2 months                 | ▼ TC (31%), LDL (32%)<br>▲ TG (125%) <sup>g</sup> | Prasad <i>et al.</i> , 1998    |
| Rabbits (n=8)                           | Flaxseed (7.5 g/kg b.w. & d), cholesterol (1%), 2 months                 | ▲ TC (33%) <sup>g</sup> , TG (40%) <sup>g</sup>   | Prasad, 1997                   |
| Apo B-1 transgenic male and female mice | Ground flaxseed (20%), cholesterol (0.1%), 2 months                      | ▼ TC (32-47%)<br>▼ hepatic TC (32%)               | Pellizzon <i>et al.</i> , 2007 |
| Hamsters <sup>f</sup> (n=12)            | Flaxseed (7.5, 15, 22.5 %), vitamin E acetate (0.34%), 4 months          | ▼ TC (17%, 19%, 23%)<br>▲ TG (54%, 64%, 45%)      | Lucas <i>et al.</i> , 2004     |
| Rabbits (n=16)                          | Phenolic complex (14.4 mg SDG/kg b.w. & d), cholesterol (0.5%), 2 months | ▼ TC (20%), LDL (14%)<br>▲ HDL (30%)              | Prasad, 2005                   |
| Rabbits (n=5)                           | SDG (15 mg/kg b.w. & d), cholesterol (1%), 2 months                      | ▼ TC (33%), LDL (35%)<br>▲ HDL                    | Prasad, 1999                   |

<sup>a</sup> Hyperlipidaemic <sup>b</sup> Postmenopausal women <sup>c</sup> n.e. No effect on TC, LDL or HDL. <sup>d</sup> Patients with the autoimmune disease *lupus nephritis*. <sup>e</sup> New Zealand white rabbits <sup>f</sup> Ovariectomised Golden Syrian female hamsters <sup>g</sup> Calculated from the article.

### *Other diseases*

Diabetes mellitus, a disorder caused by defects in insulin secretion, insulin sensitivity, or both, is characterised by hyperglycaemia and it is followed by different complications in the vascular system and in some tissues and organs (Bouché *et al.*, 2004). In experimental animal models of diabetes, a preventive or delaying effect of SDG on the development of diabetes mellitus has been obtained (Prasad, 2000, 2001). A few studies on glucose metabolism have been performed with flaxseed or the phenolic complex. Postmenopausal women ( $n=25$ ) with hypercholesterolaemia given flaxseed showed reduced glucose and insulin levels (Lemay *et al.*, 2002). In humans with hypercholesterolaemia, the phenolic complex had a reducing effect on fasting plasma glucose levels (Zhang *et al.*, 2007). In another human intervention study using type 2 diabetic hypercholesterolaemic postmenopausal women ( $n=30$ ), the subjects showed modest improvements in long-term glycaemic control, measured as reduction in glycosylated haemoglobin, after eating lower amounts of the phenolic complex for eight weeks, but there was no effect on fasting glucose and insulin sensitivity (Pan *et al.*, 2007). Another flaxseed component, the mucilage given to young healthy humans, has previously been shown to reduce postprandial glucose levels in blood plasma (Cunnane *et al.*, 1993). These studies indicate that SDG and/or other component/s in flaxseed might have lowering effects on glucose levels.

Flaxseed and SDG are suggested to protect against renal diseases (reviewed by Ranich, Bhathena & Velasquez, 2001). Renal function in animal models or in humans has been shown to improve with flaxseed treatment (Hall *et al.*, 1993; Clark *et al.*, 2001; Velasquez *et al.*, 2003) or SDG (Clark *et al.*, 2000).

## Objectives

- To develop a new HPLC method for the analysis of the phenolic glucosides (SDG, *p*-coumaric acid glucoside and ferulic acid glucoside) in flaxseed. **Paper I**
- To use the optimised direct alkaline hydrolysis method in the analysis of different flaxseed samples in Sweden. **Paper I**
- To analyse different types of bread containing flaxseed for their content of the phenolic glucosides. **Paper II**
- To investigate the composition and properties of the dioxane/ethanol-extracted phenolic complex from flaxseed. **Paper III**
- To measure the hydrogen-donating abilities of the flaxseed phenolic complex and SDG using the DPPH inhibition method. **Paper III**
- To test the effect of isolated SDG and the major SDG-HMGA-oligomers from flaxseed on tocopherols and cholesterol levels in a Sprague-Dawley rat model. **Papers IV, V**

## Materials and methods

### Samples

A pressed flaxseed cake (Alternativ Förfärdling, Glanshammar, Sweden) was milled (Retsch type ZM 1, Haan, Germany, 0.5 mm screen), and defatted twice with *n*-hexane (1:4, w/v) to obtain defatted flaxseed flour (DFF), which was used in **Papers I, III and IV**. SDG was isolated essentially as described by Johnsson *et al.* (2000) and full descriptions of the isolation are available in **Papers III-V**. Flaxseed samples, obtained from Hushållningssällskapet (harvest 2001, Örebro, Sweden) and Svalöf Weibull (harvest 2000, Svalöv, Sweden), were disintegrated and defatted in steel tubes using a method by Appelqvist (1967) which is further described in **Paper I**. Different soft and crisp breads containing flaxseed were obtained from major food outlets. Representative parts of breads were cut into smaller pieces, freeze-dried and milled (0.5 mm sieve, Retsch type ZM 1) as described in **Paper III**.

### Direct alkaline hydrolysis of flaxseed

A new method to analyse the phenolic glucosides (SDG, *p*-coumaric acid glucoside and ferulic acid glucoside) in flaxseed was developed using direct alkaline hydrolysis (Figure 5). Optimal conditions for direct alkaline hydrolysis were established using full factorial experimental designs; firstly by studying the effect of temperature and concentration of alkali, and secondly by studying the matrix effects of the internal standard and amount of DFF. Time of hydrolysis was optimised and the internal standard *o*-coumaric acid was used. The repeatability of the method was evaluated. More information is available in **Paper I**.

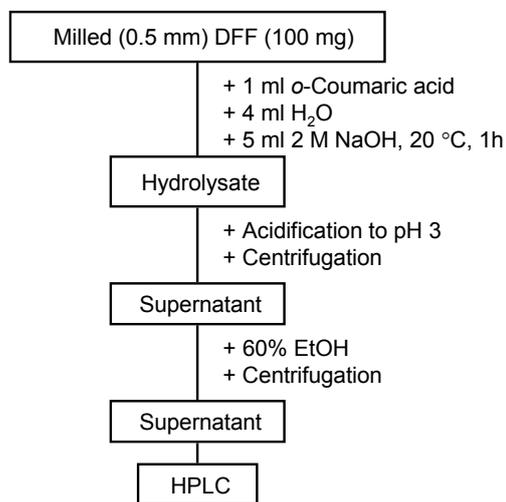


Figure 5. Diagram of the direct alkaline hydrolysis method used for analysis of phenolic glucosides in flaxseed.

## Phenolic glucosides in bread containing flaxseed

The content and relative ratios of the phenolic glucosides were analysed in freeze-dried milled bread samples by mixing with the internal standard *o*-coumaric acid and extraction with dioxane/ethanol for 48 h essentially as described by Johnsson *et al.* (2000). The analysis is fully described in **Paper III**.

## Chromatographic fractionation of oligomeric extract from flaxseed

Dioxane/ethanol extract of DFF was fractionated with C18 solid-phase extraction in a Büchner funnel using different concentrations of aqueous methanol (50, 60, 70%) to obtain the fractions F50, F60 and F70 in **Paper III** and **IV**. F50, F60 and F70 were further fractionated on Sepharose CL-6B using isocratic 80% EtOH and divided into fractions that were analysed for the content and relative ratios of the phenolic glucosides after alkaline hydrolysis by RP-HPLC as described below (Figure 6).

F50 was fractionated on Sephadex LH-20 according to hydrophobicity using isocratic 20% EtOH followed by 95% EtOH and fractions were collected and divided into four fractions (H1, H2, H3, H4) to be further fractionated on Sephadex LH-20 according to size by gel filtration using isocratic 80% EtOH **Paper III**. Each H-fraction was divided into 4 subfractions (G1, G2, G3, G4). All 16 fractions were analysed for the content and relative ratios of the phenolic glucosides after alkaline hydrolysis by RP-HPLC. This fractionation is presented schematically in Figure 6.

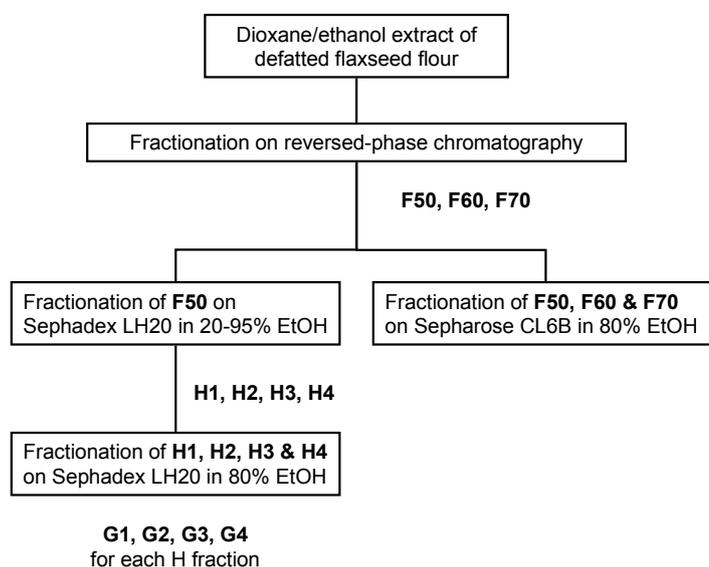


Figure 6. Diagram showing fractionation of a dioxane/ethanol extract from flaxseed.

## High-performance liquid chromatography

The contents of the phenolic glucosides and the phenolic complex were determined by HPLC on a Dionex PDA-100 (Dionex, Sunnyvale, CD, USA) system with a UV-Vis diode array detector and Chromelion software as described in **Papers I-V**. All bread samples, except three, were analysed according to **Paper I**. Due to interfering peaks, the three bread remaining samples were analysed using different gradients of mobile phase described in **Paper II**. The separation of the phenolic complex in fractions F50, F60 and F70 was performed using different gradients in the mobile phases described in **Paper III**. Calibration curves were created for *p*-coumaric acid, ferulic acid, SDG and the internal standard *o*-coumaric acid to be used in **Papers I-V**.

## Hydrogen donation ability

The ability of F50, F60, F70, SDG, ferulic acid and  $\alpha$ -tocopherol to transfer hydrogen atoms to 1,1-diphenyl-2-picrylhydrazyl (DPPH) was studied using a modified method of Brand-Williams, Cuvelier & Berset (1995). The ability of the antioxidants to transfer phenolic hydrogen atoms to DPPH was expressed as the percentage inhibition of DPPH after 30 minutes against the molar amount of SDG in the oligomeric fractions and SDG in **Paper III**.

## Animal studies

Sprague-Dawley rats arrived at 21 days of age (B&K Universal AB, Sollentuna, Sweden) and were housed individually in Macrolon IV cages in conditions described in **Papers IV** and **V**. The experiments were carried out in accordance with the guidelines and approval of the Ethical Committee for Animal Experiments in the Uppsala region. Animals were divided into groups with similar mean body weight and given an accommodation period of 4 days in **Paper IV** and 7 days in **Paper V**. After the accommodation period, the rats were given their respective diet for 27 days. The composition of the control diet and mixing of isolated SDG into the control diet is fully described in **Papers IV** and **V**. Food consumption was monitored daily, and body weights were measured weekly and at time of putting to death. Procedures during put to death of animals, and collection of samples are described in **Papers IV** and **V**. In **Paper IV**, liver samples used in the cholesterol and tocopherol analyses were immediately put in isopropanol on ice and stored at -70 °C until further analysis. In **Paper V**, liver samples used in the cholesterol and tocopherol analyses were snap-frozen in liquid nitrogen and stored in -70 °C until further analysis.

## Analysis of cholesterol and tocopherols in rat tissues

Triacylglycerols and cholesterol were quantified in plasma (Seigler & Wu, 1981) and tocopherols in plasma were quantified as described by Frank *et al.* (2003). Liver cholesterol and tocopherol samples were quantified according to Frank *et al.* (2003) in **Paper IV**, and according to a modified method by Podda *et al.* (1996) in **Paper V**, which is described in **Paper V**.

## Results and comments

### Method development (Paper I)

An easy to use HPLC method based on direct alkaline hydrolysis was developed to analyse the phenolic glucosides (SDG, *p*-coumaric acid glucoside and ferulic acid glucoside) in flaxseed (Figure 5). Extraction of the phenolic complex with polar solvents was excluded and the internal standard *o*-coumaric acid was used. Minor effects of temperature and concentration of alkali were observed on the yield of the phenolic glucosides. Optimal conditions for direct alkaline hydrolysis were established as 1 M of NaOH at 20 °C for one hour of hydrolysis and polysaccharides and protein were precipitated using 60% aqueous ethanol. The recovery of the internal standard was studied with different levels of DFF. Pinoresinol and matairesinol did not agree with any of the chromatographic peaks obtained using this method. Degradation of matairesinol under alkaline conditions has been observed previously (Milder *et al.*, 2004) and might explain why matairesinol was not detected with this method. Direct alkaline hydrolysis resulted in a higher yield of (+)-SDG in DFF than obtained with alkaline hydrolysis after dioxane–ethanol extraction of DFF. Good repeatability of the method was obtained by two analysts.

### Content of phenolic glucosides in flaxseed (Paper I)

Flaxseed samples ( $n=27$ ) from two locations in Sweden were analysed using direct alkaline hydrolysis. A high variation in the content of phenolic glucosides was obtained (Figure 7). The content of (+)-SDG obtained in this study ranged from 1.2-2.6% in flaxseed and was found to be higher than levels reported by other authors, as summarised in Table 1. The variety Jupiter contained most phenolic glucosides and Niagara S least. Weak correlations were found between SDG and hydroxycinnamic acid glucosides.

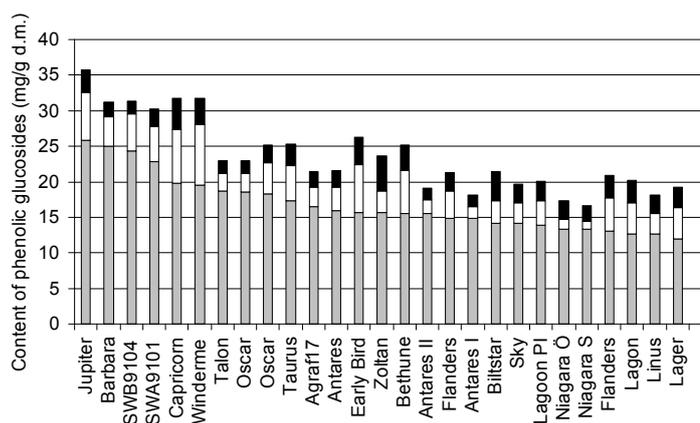


Figure 7. Content of phenolic glucosides (SDG, *p*-coumaric acid glucoside and ferulic acid glucoside) in Swedish flaxseed samples analysed by direct alkaline hydrolysis.

## Content of phenolic glucosides in bread containing flaxseed (Paper II)

The flaxseed content in seventeen breads ranged from 1.5 to 9 g/100 g fresh bread. This flaxseed content is within the advised intake of flaxseed by the National Food Administration in Sweden. Alkali mixed directly with bread samples containing high amounts of starch resulted in gelatinisation of starch and increased viscosity of the samples, which complicated further sample treatment. Therefore, the bread samples first had to be extracted with dioxane/ethanol and secondly hydrolysed with alkali. An interfering peak with the internal standard *o*-coumaric acid was observed that might derive from other ingredients mixed into these breads. However, altering the gradient of the mobile phase separated the peaks.

Variation in the content of the phenolic glucosides in the soft and crisp bread was observed (Figure 8). The major phenolic glucoside was SDG, ranging from 7.6 to 105 mg/100 g dry bread. The content of SDG in flaxseed bread products in this study was within the range obtained by other authors, as summarised in Table 2. Less abundant phenolic glucosides in the breads were *p*-coumaric acid glucoside (3.3-33 mg/100 g dry bread) and ferulic acid glucoside (3.3-18 mg/100 g dry bread).

Strong positive correlations were obtained between SDG and the hydroxycinnamic acid derivatives, as well as between *p*-coumaric acid glucoside and ferulic acid glucoside. These correlations indicate that the bread ingredients and the bread-making conditions only have a small impact on the proportion of the phenolic glucosides in the breads.

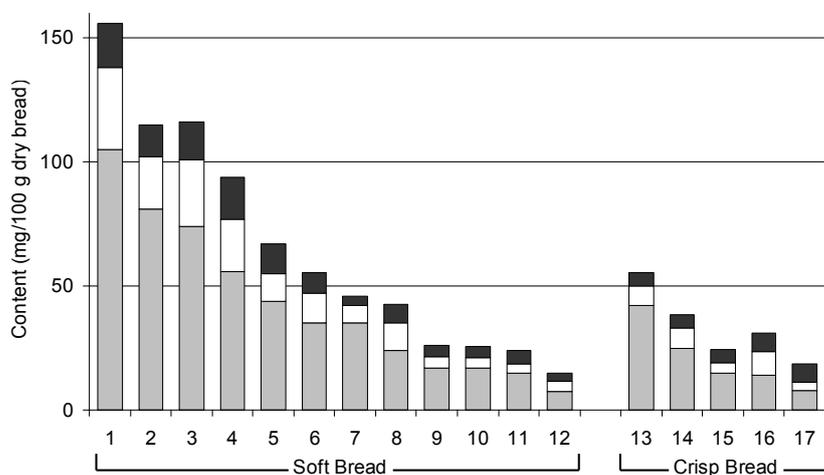


Figure 8. Content (mg/100g dry bread) of the phenolic glucosides (SDG ■, *p*-coumaric acid glucoside □ and ferulic acid glucoside ■) in 12 soft breads and 5 crisp breads.

### Structural Features (Paper III)

A dioxane/ethanol extract from flaxseed containing the phenolic complex was fractionated into three oligomeric fractions (F50, F60 and F70) by reversed-phase chromatography and further subfractionated by Sepharose CL-6B (Figure 6). The F50 fraction, which had the highest proportion of hydroxycinnamic acid glucosides, was further fractionated on Sephadex LH-20 according to hydrophobicity (H1-H4) and size (G1-G4).

#### *Fractionation by reversed-phase chromatography*

Flaxseed oligomers were fractionated into fractions with distinctively different hydrophobic properties and composition of the main phenolic glucosides. The major fraction, F60, had a relative composition of the phenolic glucosides similar to that of DFF (Figure 9). The minor fraction, F50, had the highest relative composition of hydroxycinnamic acid derivatives. Reversed-phase chromatography separated the oligomers according to polarity, which related to the composition of the phenolic glucosides. The most polar fraction F50 contained proportionally less SDG and the least polar fraction F70 contained proportionally more SDG.

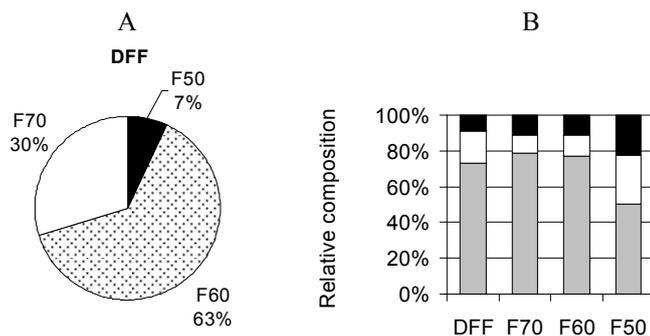


Figure 9. A. Relative proportions of F50, F60 and F70 in DFF, calculated from the total content of the phenolic glucosides (SDG, *p*-coumaric acid glucoside and ferulic acid glucoside). B. Relative composition of SDG ■, *p*-coumaric acid glucoside □, and ferulic acid glucoside ■ in F50, F60 and F70 from flaxseed obtained by reversed-phase chromatography, and the relative composition of the phenolic glucosides in defatted flaxseed flour (DFF) analysed with direct alkaline hydrolysis (Paper III).

#### *Fractionation by Sepharose CL-6B*

Fractionation of F50, F60 and F70 on Sepharose CL-6B resulted in a wide range of molecular sizes for F60 and F70. The relative composition of phenolic glucosides in the collected fractions of F50, F60 and F70 was stable and was not effected by the gel filtration fractionation. The fractions were partly separated by size-exclusion and partly by an unknown chromatography, which was observed by a large proportion of UV-absorbing material eluting after the total volume of the column. Hydrogen bonding between the stationary phase, unknown compounds of

hydrophobic nature in the oligomers and/or other structural features of the oligomers might result in this unknown chromatography.

*Fractionation of F50 on Sephadex LH-20*

F50 with the highest proportion of hydroxycinnamic acid glucosides was fractionated on Sephadex LH-20 with the intention of separating on the basis of hydrophobicity and of further separating the subfractions on the basis of size using different mobile phases of ethanol concentrations, but both mobile phases fractionated according to both size and hydrophobicity. The results indicate that F50 contains low molecular size oligomers with less hydrophobic properties and different proportions of the phenolic glucosides than F60 and F70.

The relative composition of the phenolic glucosides in the 16 fractions is compared in Figure 10. In contrast to the results from reversed-phase chromatography, the proportion of SDG decreased with increasing hydrophobicity. The small size oligomers (G4) had larger variation in the relative composition than large size oligomers (G1). In the least hydrophobic oligomers (H1), the proportion of SDG increased somewhat with decreasing size of oligomer. In the more hydrophobic oligomers (H2-H4), the relative ratio of SDG decreased with decreasing size of oligomer.

Fractions H1G1 and H1G2 were of high molecular size with least hydrophobic properties. Furthermore, these fractions were the largest fractions of F50, with the most similar relative phenolic glucoside compositions to F50 (Figures 10 & 11). Among the most hydrophobic oligomers, G4H4 was among the smallest fractions of F50 with the smallest oligomers, and the highest relative content of hydroxycinnamic acid glucosides compared with SDG.

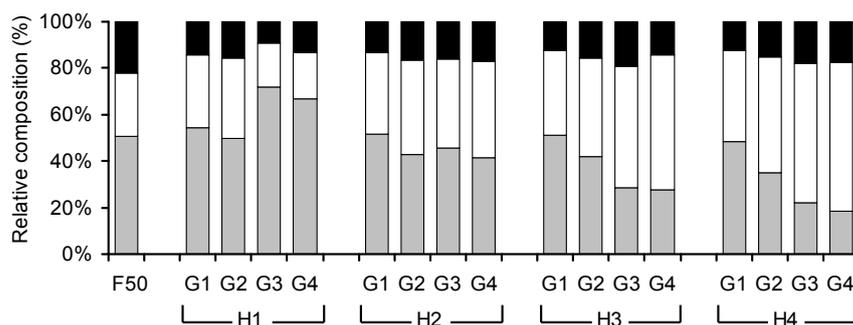


Figure 10. Fractionation of F50 on Sephadex LH-20 according to hydrophobicity (H1, H2, H3, H4) and gel filtration (G1, G2, G3, G4). The phenolic compounds in each fraction were SDG ■, p-coumaric acid glucoside □, and ferulic acid glucoside ■.

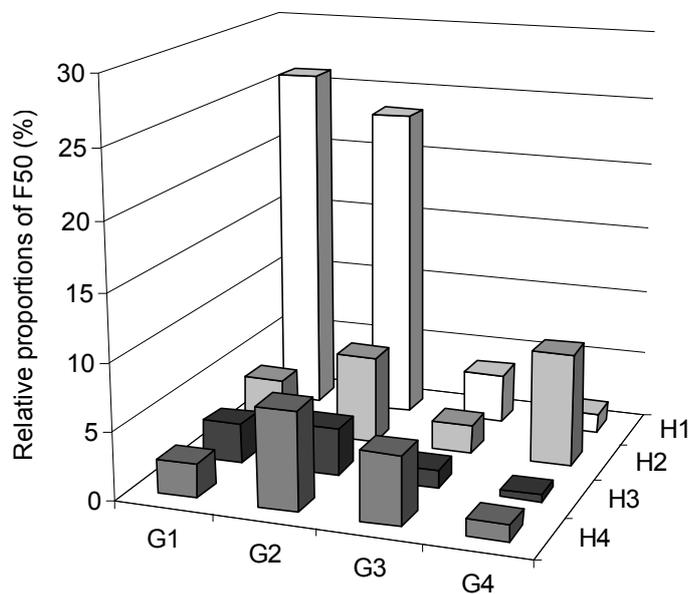


Figure 11. Fractionation of F50 on Sephadex LH-20 according to hydrophobicity (H1, H2, H3, H4) and gel filtration (G1, G2, G3, G4). The relative proportions (%) of the 16 fractions obtained from F50.

The inability to separate SDG and the hydroxycinnamic acid glucosides during the chromatography of F50, F60 and F70 in **Paper III** suggests connections or overlap between the SDG-HMGA oligomers and the hydroxycinnamic glucosides in their oligomeric structures. The hydroxycinnamic acid glucosides and ferulic acid residues have been shown to be connected directly to SDG but no linkage between HMGA and the hydroxycinnamic acid glucosides was detectable by NMR or MS analysis (Struijs *et al.*, 2008). Those authors suggested that the hydroxycinnamic acid glucosides are not connected to HMGA and that they are terminal units of the SDG complex. However, it is not known whether some hydroxycinnamic acid glucosides unconnected to SDG might exist in the phenolic complex. Results obtained by Struijs *et al.* (2008) and in **Paper III** provide new knowledge on the structures and properties of the phenolic complex in flaxseed.

### Hydrogen-donating ability using DPPH (Paper III)

The hydrogen-donating abilities of SDG, F50, F60, and F70 were studied using DPPH in comparison with ferulic acid and  $\alpha$ -tocopherol (Figure 12). The reduction of DPPH (%) by SDG, F50, F60 and F70 was similar and consistent with their molar levels of SDG, confirming that the hydroxycinnamic acid derivatives, in which the phenolic groups are blocked by glucosylation, were not active as antioxidants. Oligomeric fractions had slightly lower hydrogen donating ability than SDG, which might be caused by steric hindrance in the oligomers. A lower ability of phenolic oligomers compared with monomers to donate hydrogens to DPPH has been reported previously (Goupy *et al.*, 2003). At lower concentrations, F50 had stronger H-donating ability than F60 and F70, which might be due to less steric hindrance or to differences in solubility. Ferulic acid had comparable and  $\alpha$ -tocopherol had lower H-donating ability than SDG.

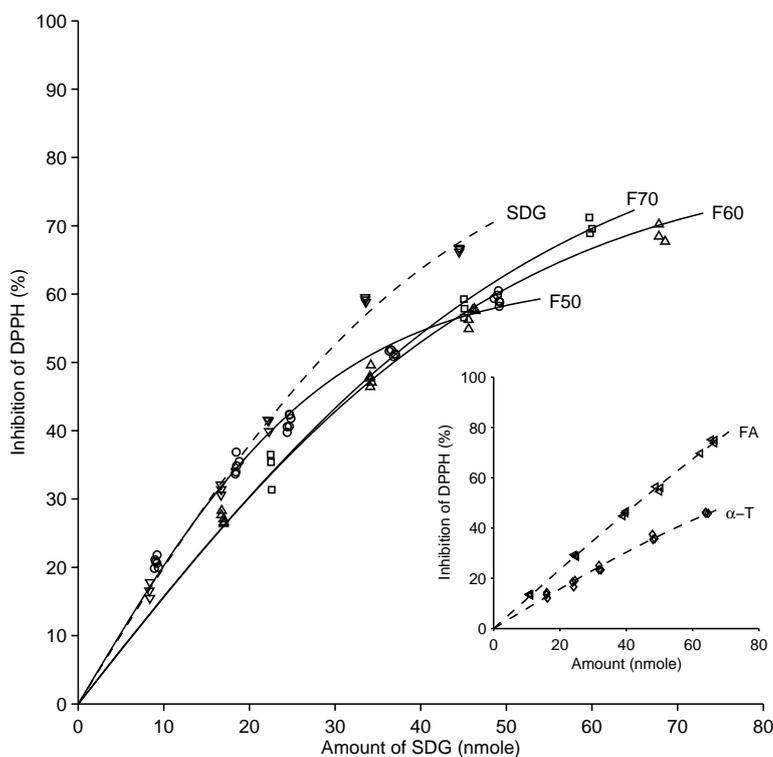


Figure 12. Inhibition of DPPH (%) after 30 minutes of incubation with SDG, F50, F60, and F70 in 80% methanol, and  $\alpha$ -tocopherol and ferulic acid in 100% methanol. The total amount of SDG in F50, F60 and F70 was calculated on a molar basis. (Kind permission of Food Chemistry)

## Bioactivities of SDG and the phenolic complex

### *SDG and its oligomers decreased vitamin E levels and increased liver cholesterol in rats (Paper IV)*

Comprehensive results from human epidemiological studies and animal tumour models suggest that  $\alpha$ -tocopherol possess chemopreventive and chemotherapeutic effect against prostate cancer (Ni & Yeh, 2007). The effect of SDG and the phenolic complex on levels of vitamin E was studied using a Sprague–Dawley rat model. Rats (3 groups) were fed a control diet and a diet containing 0.1% SDG or the phenolic complex containing 0.1% SDG for 27 days. Groups showed no differences in feed intake, animal body weight or liver weight at the end of the experiment. SDG and the phenolic complex significantly reduced  $\alpha$ - and  $\gamma$ -tocopherols in rat plasma and liver (Figure 13A&B). The contribution of *p*-coumaric glucoside and ferulic acid glucosides to this effect was negligible due to the equal effect of SDG and the phenolic complex. SDG and the phenolic complex had no significant effect on plasma triacylglycerols and cholesterol but caused a slight but significant elevation of liver cholesterol and percentage of cholesterol in the liver lipids (Figure 13C&D).

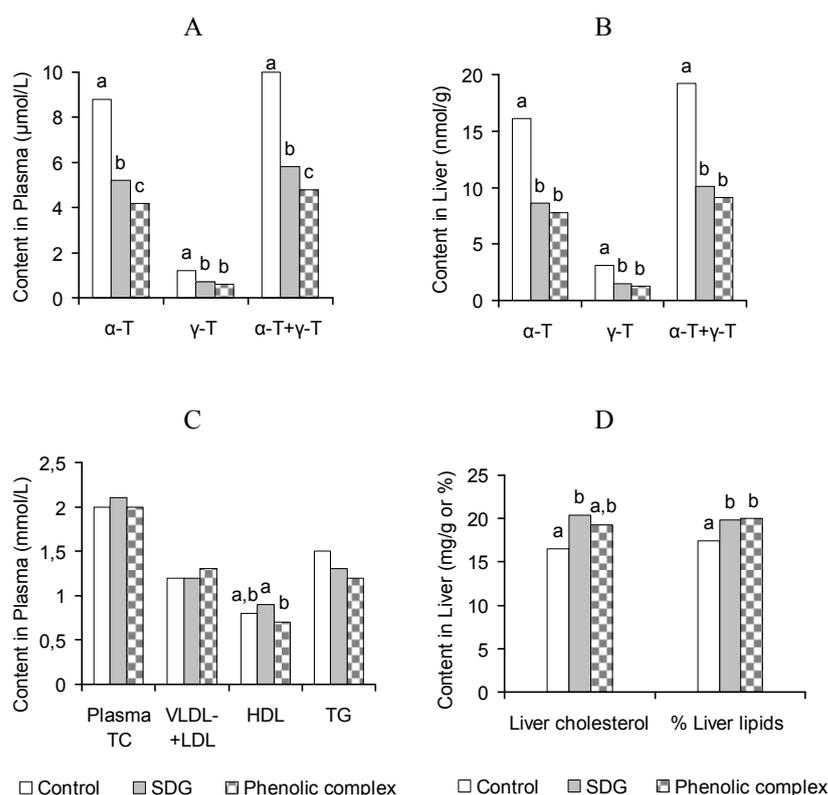


Figure 13. Effects of secoisolariciresinol diglucoside (SDG) and the phenolic complex on  $\alpha$ - and  $\gamma$ -tocopherol levels in rat plasma (A) and liver (B), and on cholesterol levels in rat plasma (C) and liver (D). Different letters indicate significant differences between the groups ( $P < 0.05$ ).

Similarly to **Paper IV**, two other animal studies have shown a reduction in tocopherol levels in rat liver. Ratnayake *et al.* (1992) obtained a reduction in  $\alpha$ - and  $\gamma$ -tocopherol levels in the liver, heart and spleen of rats given flaxseed. When hydroxymatairesinol (HMR) is metabolised in the colon of humans and rats, it is converted to ED, EL and matairesinol. Given to rats, it was shown to reduce  $\gamma$ -tocopherol levels in rat liver (Yamashita *et al.*, 2007). Tocopherols are metabolised in the liver by cytochrome P450s, similarly to xenobiotics, and excreted in the urine as carboxyethyl hydroxychromans (CEHCs) (Sontag & Parker, 2002). A non-significant increase in  $\gamma$ -CEHC in the urine of rats consuming HMR might indicate increased cytochrome P450 (CYP450) metabolism of  $\gamma$ -tocopherol in the liver (Yamashita *et al.*, 2007). Aromatic oxidative metabolites of ED and EL obtained in urine suggest an oxidative metabolism by CYP450 (Jacobs & Metzler, 1999; Niemeyer & Metzler, 2002). Human pregnane X receptor (PXR) involved in the metabolism of CYP3A substrates in liver and intestinal tissues is moderately activated by EL *in vitro* (Jacobs, Nolan & Hood, 2005), which might lead to a reduction in vitamin E levels.

*SDG had no effect on vitamin E and cholesterol in rats (Paper V)*

Dose-dependent effects of SDG on the levels of vitamin E and cholesterol in plasma and liver were studied using a Sprague–Dawley rat model. Rats (5 groups) were given a daily control diet containing 0 (control), 0.1, 0.05, 0.025 and 0.0125% SDG isolated from flaxseed, respectively, for 27 days. Groups showed no differences in feed intake, animal body weight or liver weight at the end of the experiment. In this study, no effect was observed for dose-dependent intake of SDG on tocopherol and cholesterol levels in rat plasma or liver. Similarly to **Paper V**, DFF (11%) given to Wistar rats on a high cholesterol (1%) diet for 4 weeks had no effect on  $\alpha$ - or  $\gamma$ -tocopherol levels in plasma and liver (Yamashita, Ikeda & Obayashi, 2003).

**Papers IV** and **V** contradict each other and previous studies on the hypocholesterolaemic effects of SDG and the phenolic complex shown in humans and rats, as summarised in Table 3. Moreover, results in **Paper V** on levels of tocopherol in rats contradict the results in **Paper IV**. The reasons for these differences in tocopherol and cholesterol levels in rats are still unexplained and further research is needed.

## Summary and conclusions

The findings of this thesis can be summarised as follow:

- A new, fast and simple HPLC method was developed based on direct hydrolysis of the defatted flaxseed flour (DFF) using alkali. This method gave higher yield than previous methods.
- Flaxseed samples grown in two locations in Sweden varied considerably in their content of (+)-SDG (11.9–25.9 mg/g), (-)-SDG (2.2–5.0 mg/g), *p*-coumaric acid glucoside (1.2–8.5 mg/g), and ferulic acid glucoside (1.6–5.0 mg/g). Analysis of different flaxseed samples confirmed that flaxseed is a rich source of SDG as previously reported.
- Bread products containing flaxseed were analysed for their content of (+)-SDG (7.6-105 mg/100 g d.w.), *p*-coumaric acid glucoside (3.3-33 mg/100 g d.w.) and ferulic acid glucoside (3.3-18 mg/100 g d.w.). Strong positive correlations between the phenolic glucosides were found, indicating no major effect of raw material or bread-making process on relative content of the phenolic glucosides in flaxseed.
- The structural features of the flaxseed oligomers revealed a considerable complexity of the components in the phenolic complex. Chromatographic fractionation suggested that oligomers have broad molecular weight distribution and variable composition of phenolic glucosides. A complicated linkage pattern and/or possible interactions with other components also seem to contribute to the observed complexity.
- SDG and oligomeric fractions showed similar hydrogen-donating abilities to ferulic acid but higher than  $\alpha$ -tocopherol in the DPPH inhibition method. SDG was suggested to be the only active antioxidant in the oligomeric fractions.
- Contradicting results were obtained in two studies on the effect of SDG from flaxseed on levels of Vitamin E and cholesterol in a Sprague-Dawley rat model.

## **Future research**

It is important to study the structure of the phenolic complex in order to further understand the absorption, metabolism and bioactivity of flaxseed lignans in humans and animals, and to further understand the biosynthesis of the phenolic complex in flaxseed. However, the structure of the phenolic complex and connections between different phenolic components are still to a large extent unknown. Molecular weight distribution, linkage patterns and interactions with other components need to be further evaluated.

Epidemiological and animal studies indicate a protective effect of flaxseed and SDG against breast cancer, prostate cancer, cardiovascular disease and possibly other diseases. This had led to a rapid growth of new flaxseed containing food products with different types of health claims. To support these health claims, more studies in humans are needed. Also the effect of SDG on bioavailability of vitamin E in humans and possible toxicological effects of SDG must be studied before its complete potential as a functional food can be fully evaluated. Many questions remain and need to be answered on the effects of SDG on the human metabolism.

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