Bioactive Phytochemicals in Flaxseed

With Particular Emphasis on the Secoisolariciresinol Oligomer

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Abstract

Flaxseed (*Linum usitatissimum* L.) is rich in health-promoting bioactive compounds. Among plant foods, flaxseed has the highest content of lignans, mainly in the form of secoisolariciresinol diglucoside (SDG). Flaxseed oil also has a very high concentration of the essential omega-3 fatty acid alpha-linolenic acid (ALA). This thesis presents studies on both SDG and ALA.

An HPLC method for quantification of SDG in hydrolysed flaxseed extracts was developed and used to compare the SDG content in 29 flaxseed cultivars (6.1-13.3 mg/g seed, dry weight). Full spectroscopic (NMR) data were obtained for two compounds, 4-O-β-D-glucopyranosyl-*p*-coumaric acid (CoAG) and 4-O-β-D-glucopyranosyl-ferulic acid, eluting before SDG in the HPLC system developed. NMR analyses provided the first evidence that SDG in flaxseed is bound in a linear oligomeric structure with an average composition of five SDG residues interlinked by four 3-hydroxy-3-methyl glutaric acid residues (average size 4 kDa). Chromatographic (UHPLC, GPC, SPE) and spectroscopic (MALDI-TOF) analyses of seven different flaxseed cultivars showed that the size of the SDG oligomers differed among cultivars depending on seed SDG/CoAG ratios.

In attempts to decrease the use of fish-based feeds in aquaculture, vegetable oils are being assessed as replacements for fish oil. However, this has hitherto resulted in decreased levels of the long-chain omega-3 fatty acids (n-3 HUFA) that make fish a healthy food choice for humans. Previous studies have shown that sesamin, a lignan from sesame seed, is able to increase conversion of ALA to n-3 HUFA in rainbow trout (*Oncorhynchus mykiss*) but less efficiently than expected when flaxseed oil, given its high ALA content, was used. In order to investigate whether high levels of ALA or possibly minor compounds in flaxseed oil could be responsible for the low n-3 HUFA formation, rainbow trout were fed diets containing purified flaxseed triacylglycerols (PFSO) with and without sesamin. However, it was found that PFSO was unable to increase ALA conversion compared with unaltered flaxseed oil and that PSFO with or without sesamin addition had the opposite effect to unaltered flaxseed oil with or without sesamin addition, respectively. The reasons for these effects are discussed.

Keywords: Lignans, HPLC, linseed, rainbow trout, alpha-linolenic acid, n-3 HUFA

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Bioaktiva fytokemikalier i linfrö - Med särskild betoning på secoisolariciresinol-oligomeren

Svensk sammanfattning

Linfrö (*Linum usitatissimum* L.) innehåller höga halter av bioaktiva ämnen. Bland födoväxter har linfrö det högsta innehållet av lignaner, främst i form av secoisolariciresinol diglukosid (SDG). Linfröolja har också bland de högsta koncentrationerna av den essentiella omega-3 fettsyran alfalinolensyra (ALA). Den här avhandlingen presenterar arbeten om både SDG och ALA.

En HPLC-metod för kvantifiering av SDG i hydrolyserade linfröextrakt utvecklades och användes för att jämföra innehållet av SDG i 29 linfrösorter (6.1–13.3 mg/g frö, torrvikt). Vidare erhölls fullständiga spektroskopiska data för två föreningar, 4-O-β-D-glukopyranosyl-*p*-coumarsyra (CoAG) och 4-O-β-D-glukopyranosyl-ferulasyra, som eluerades före SDG i den framtagna HPLC-metoden. Med hjälp av olika NMR-tekniker lades de första bevisen fram för att SDG i linfrö var bundet i en linjär oligomer struktur innehållande ett genomsnitt av fem SDG-enheter sammanlänkade av fyra 3-hydroxy-3-metylglutarsyror (medelvikt 4 kDa). Genom kromatografiska (UHPLC, GPC, SPE) och spektroskopiska (MALDI-TOF) jämförelser av sju linfrösorter visades att SDG-oligomerer har olika storlekar i olika linfrösorter beroende på fröernas SDG/CoAG-kvot.

I och med ansträngningar att minska användningen av fiskbaserade foder i fiskodlingen görs försök att ersätta fiskolja i foder med vegetabiliska oljor. Det har dock hitills resulterat i minskade halter av de långkedjiga omega-3 fettsyror (n-3 HUFA) som gör fisk till ett hälsosamt livsmedelsval för människor. Tidigare studier har visat att en lignan från sesamfrö, sesamin, har förmågan att öka omvandlingen av ALA till n-3 HUFA i regnsbågslax (*Oncorhynchus mykiss*) men, med tanke på linfröoljans höga innehåll av ALA, i mindre grad än väntat när linfröolja användes i fodret. För att undersöka huruvida den höga ALA-halten eller möjligen de ”småkomponenter” som finns i linfröolja orsakar den låga ALA-omvandlingen, utfodrades regnbågslax med triacylglycerolfraktionen (PFSO) från linfröolja med eller utan tillsats av sesamin. Det visade sig dock att PFSO inte ökade ALA-omvandlingen jämfört med hel linfröolja samt att PFSO med eller utan tillsatt sesamin, resulterade i omvända effekter jämfört med linfröolja med respekive utan tillsatt sesamin. Orsakerna till dessa effekter diskuteras.

Hur kan det finnas så mycket att titta på i ett litet linfrö?
Momma

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Johnsson, P., Kamal-Eldin, A., Lundgren, L.N., Åman, P. (2000). HPLC method for analysis of secoisolariciresinol diglucoside in flaxseeds. *Journal of Agricultural and Food Chemistry* 48, 5216-5219.

II Johnsson, P., Peerlkamp, N., Kamal-Eldin, A., Andersson, R.E., Andersson, R., Lundgren, L.N., Åman, P. (2002). Polymeric fractions containing phenol glucosides in flaxseed. *Food Chemistry* 76, 207-212.

III Kamal-Eldin, A., Peerlkamp, N., Johnsson, P., Andersson, R., Andersson, R.E., Lundgren, L.N., Åman, P. (2001). An oligomer from flaxseed composed of secoisolariciresinol diglucoside and 3-hydroxy-3-methyl glutaric acid residues, *Phytochemistry* 58, 587-590.

IV Johnsson, P., Vincken, J-P., Gruppen, H., Kamal-Eldin, A. The size of the lignan oligomer in flaxseed is influenced by its monomeric constituents (manuscript).

V Johnsson, P., Brännäs, E., Kamal-Eldin, A., Pickova, J. The effect of purified flaxseed triacylglycerols and sesamin on fatty acid composition in white muscle of rainbow trout (*Oncorhynchus mykiss*) (manuscript).

Papers I-III are reproduced with the permission of the publishers.

The contribution of Pernilla Johnsson to the papers included in this thesis was as follows:

I Took main responsibility for the analytical work and for preparation and revision of the paper.

II Took part in planning the study, had main responsibility for the analytical work and assisted in preparation and revision of the paper.

III Took part in planning the study, the analytical work and preparation and revision of the paper.

IV Took a major part in planning the work and had main responsibility for the analytical work and for preparation of the manuscript.

V Took a major part in planning the work and had main responsibility for the analytical work and for preparation of the manuscript.

Abbreviations

AA	Arachidonic acid (20:4 n-6)
ALA	alpha-linolenic acid/α-linolenic acid (18:3 n-3)
aq.	Aqueous
^{13}C NMR	Carbon NMR
CoAG	*p*-coumaric acid glucoside
COSY	Correlation spectroscopy (NMR)
DFF	Defatted flaxseed flour
DHA	Docosahexaenoic acid (22:6 n-3)
DPA	Docosapentaenoic acid (22:5 n-3)
EPA	Eicosapentaenoic acid (20:5 n-3)
FeA	Ferulic acid
FeAG	Ferulic acid glucoside
FSO	Flaxseed oil
FSO+S	Flaxseed oil + sesamin
GC	Gas chromatography
GLM	General Linear Model
GPC	Gel permeation chromatography
HDG	Herbacetin diglucoside
HMBC	Heteronuclear multiple bond correlation (NMR)
HMGA	3-hydroxy-3-methylglutaric acid
^{1}H NMR	Proton NMR
HPLC	High performance liquid chromatography
HSQC-DEPT	Heteronuclear single quantum coherence - Distortionless enhancement by polarization transfer (NMR)
HUFA	Highly unsaturated fatty acids (≥ 20 carbons and ≥ 3 double bonds)
LA	Linoleic acid (18:2 n-6)
LC-MS	Liquid chromatography mass spectrometry

MALDI-TOF MS	Matrix Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry
MO	Mixed oil (sunflower + linseed oil, 6:4 v/v)
MO+S	Mixed oil + sesamin
MS	Mass spectrometry
MW	Molecular weight (g/mol or Da)
NMR	Nuclear magnetic resonance spectroscopy
PFSO	Purified flaxseed oil
PFSO+S	Purified flaxseed oil + sesamin
PL	Phospholipid
SAS	Statistical Analysis System for Windows
SD	Standard deviation
SDG	Secoisolariciresinol diglucoside
SECO	Secoisolariciresinol
SPE	Solid phase extraction
TAG	Triacylglycerol
TOCSY	Total correlation spectroscopy (NMR)
UHPLC	Ultra-high performance liquid chromatography

1 Introduction

Archaeological findings suggest that flax (*Linum usitatissimum* L.) has been used by humans for about 10,000 years. It was probably one of the first plants to be domesticated, around 6,000 years B.C. in Mesopotamia, as evidenced by findings of large flaxseeds resulting from irrigation of cultivated land. There are records of flaxseed being used in bread in Jordan and Greece 3,000 years ago. Flaxseed and flaxseed oil have been valued as foods and for medical purposes since ancient times (Vaisey-Genser and Morris, 2003). Flaxseed is now attracting increased interest as a food due to its high fibre content, high content of bioactive phenolic compounds (mainly lignans) and high content of the essential omega-3 fatty acid alpha-linolenic acid (ALA) (Dean, 2003). However, in many countries there are restrictions on the amount of flaxseed that may be included in foods (Dean, 2003) due to its content of cyanogenic compounds. The Swedish National Food Administration advises consumers to not eat more than two tablespoons of flaxseed per day, but an occasional intake of 3-10 tablespoons is not considered to entail any acute health risks (Livsmedelsverket, 2009). The five leading producers of flaxseed world-wide are, in decreasing order: Canada, China, India, USA and Ethiopia (FAOSTAT, 2007).

Flaxseed is also sometimes called linseed. In North America, the names 'flaxseed' and 'linseed' are used interchangeably, although there is a tendency to use the name flaxseed for seeds that are used for human consumption. In Europe, the name flax is used for plants that are grown for linen fibre, while linseed refers to seeds that are grown for technical and nutritional purposes (Vaisey-Genser and Morris, 2003). The term flaxseed is used throughout this thesis, which focuses on the two traits of flaxseed that have gained most attention during recent decades, namely its high lignan and ALA content.

1.1 Flaxseed lignans

In the 1970s, a class of compounds called lignans, especially abundant in fibre-rich parts of plants, was recognised by the National Cancer Institute (USA) as high-interest compounds with regard to antitumour activity (Hartwell, 1976; Barclay, 1976). A few years later, the enterolignans (also called mammalian lignans) enterolactone and enterodiol, which were identified as metabolic products of dietary plant origin, were discovered in human urine (Setchell *et al.*, 1979; Setchell *et al.*, 1980b; Stich *et al.*, 1980; Setchell *et al.*, 1981; Axelson *et al.*, 1982). Their excretion in urine from human and vervet monkey females was shown to vary during the reproductive cycle, with a maximum during the luteal phase and early pregnancy. Their excretion was negatively correlated to that of oestrogen, suggesting a physiological function relating to hormone metabolism (Setchell *et al.*, 1979; Stich *et al.*, 1980; Setchell *et al.*, 1980a; Setchell *et al.*, 1981; Adlercreutz, 1984; Horwitz and Walker, 1984). This eventually led to the suggestion that fibre-rich plant foods containing hormone-like compounds such as lignans and isoflavonoids, also known as phyto-oestrogens, may influence oestrogen metabolism and reduce the incidence of breast and colon cancer and possibly other diseases (Setchell *et al.*, 1981; Adlercreutz, 1982, 1984; Horwitz and Walker, 1984; Adlercreutz, 1990). In plants, lignans are thought to act as growth regulators or as a defence against external threats (Ayres and Loike, 1990). The number of lignans identified is steadily increasing. Lignans come in a large number of structural variations and they have been shown to possess a wide variety of biological activities such as antitumour, antimitotic, inhibition of DNA and RNA synthesis, antiviral, inhibition of enzyme activity, anti-insect, antimicrobial and fungistatic (MacRae and Towers, 1984), which is why they are attracting much interest in research.

Dietary sources of lignans include seeds, legumes, cereals, vegetables, berries, seaweed, tea and alcoholic beverages (Thompson *et al.*, 1991; Namiki, 1995; Mazur *et al.*, 1996; Mazur, 1998; Mazur and Adlercreutz, 1998; Mazur *et al.*, 1998a, b; Liggins *et al.*, 2000; Mazur *et al.*, 2000; Nurmi *et al.*, 2003). Quantitative data on some lignans present in food are presented in Table 1. Flaxseed and sesame seed contain tens to thousands of times more lignans than most other edible plants (Table 1) and can therefore contribute significantly to lignan intake through the diet, even with moderate intakes.

Table 1. *Examples of lignan levels determined for some selected food plant products*

Source	Lignan	Level (ppm[1], fresh weight)	Reference
Flaxseed	Secoisolariciresinol[2]	6285-13680[3]	Eliasson *et al.*, 2003
Sesame seed	Sesamin	70-7120	Moazzami and Kamal-Eldin, 2006
Sunflower seed	Lariciresinol	7	Milder *et al.,* 2005
Rye	Syringaresinol	35	Smeds *et al.*, 2007
Wheat	7-hydroxymatairesinol	28	Smeds *et al.*, 2007
Broccoli	Lariciresinol	10	Milder *et al.*, 2005
Strawberry	Pinoresinol	2	Milder *et al.*, 2005
Tea (blend)	Pinoresinol	0.3	Milder *et al.*, 2005

[1]Parts per million

[2]Recalculated from secoisolariciresinol diglucoside

[3]Dry weight

Plant lignans are phenolic secondary metabolites with a basic structure consisting of two interlinked phenylpropanoid molecules (Figure 1a). There are two major classes of lignans, the dibenzylbutane lignans and the furanofuran lignans, of which secoisolariciresinol diglucoside (SDG) from flaxseed and sesamin from sesame seed are respective examples (Figure 1b and c). The prevailing lignan in flaxseed is secoisolariciresinol diglucoside (SDG; MW = 686.7 Da; Figure 1b) (Bakke and Klosterman, 1956; Chimichi *et al.*, 1999; Sicilia *et al.* 2003), which is often referred to as secoisolariciresinol or SECO, the aglycone of SDG (Mazur *et al.*, 1996) depending on the method of analysis. In flaxseed, SDG exists in two isomeric forms, (+)-SDG and (-)-SDG, of which (+)-SDG is the major isomer (Bambagiotti-Alberti *et al.* 1994; Eliasson *et al.*, 2003). Smaller quantities of matairesinol, isolariciresinol, lariciresinol, demethoxy-secoisolariciresinol and pinoresinol have also been identified in flaxseed (Meagher *et al.*, 1999; Sicilia *et al.*, 2003). The lignan content in flaxseed differs between varieties, but has also been shown to depend on growing location and year (Westcott and Muir, 1996b; Thompson *et al.*, 1997).

There are only a few studies on the stability of SDG through food processing. The SDG content in bakery products, added as a supplement or present in flaxseed, has been shown to withstand normal baking procedures (Westcott and Muir, 1996b; Muir and Westcott, 2000; Hyvärinen *et al.*, 2006), and storage of bread for 1 week at room temperature or 1-2 months at -25 °C (Hyvärinen *et al.*, 2006 a).

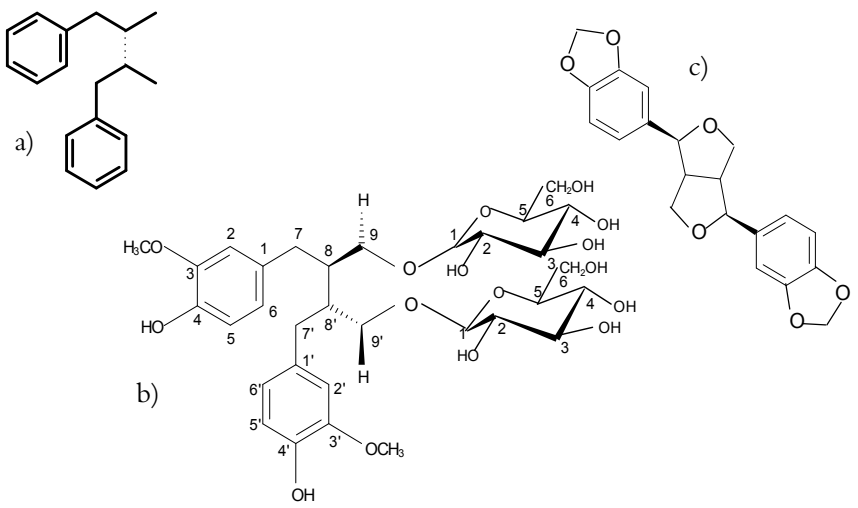

Figure 1. a) The basic structure of lignans consisting of two interlinked phenylpropanoid molecules, b) secoisolariciresinol diglucoside, a dibenzylbutane lignan, c) sesamin, a furanofuran lignan.

Strandås *et al.* (2008b) showed that the level of SDG in 17 breads containing flaxseed was in the expected range considering the amount of flaxseed added. They also showed that the SDG and the hydroxycinnamic acids deriving from the flaxseed in the bread correlated to each other in a similar way as they do in pure flaxseed. From these results it was concluded that SDG and hydroxycinnamic acids from flaxseed are not affected by bread-making conditions (Strandås *et al.*, 2008b). Added SDG has also been reported to be stable during heat treatment, fermentation and storage of dairy products such as yoghurt and ripened hard cheese, with the exception of whey drinks where a 25% loss occurred after 6 months of storage (Hyvärinen *et al.*, 2006b).

As well as lignans, flaxseed also contains other phenolic compounds such as phenolic acids and flavonoids. Free or bound phenolic acids that have been identified in flaxseed include *p*-coumaric acid, ferulic acid, caffeic acid, *p*-hydroxybenzoic acid, gentisic acid, salicylic acid, sinapic acid, syringic acid and vanillic acid (Kozlowska *et al.*, 1983; Dabrowski and Sosulski, 1984). Glucosides of *p*-coumaric (CoAG), ferulic (FeAG) and caffeic acid have been detected in flaxseed (Westcott and Muir, 1996a; Westcott and Muir, 2000; Eliasson *et al.*, 2003; Struijs *et al.*, 2007). Linocinnamarin (4-O-β-D-glucopyranosyl-cinnamate; *p*-coumaric acid glucoside) was first isolated from flaxseed by Klosterman *et al.* (1955). The compound linusitamarin (methyl

3-β-D-glucopyranosyl-5-methoxycinnamate) was identified by Luyengi *et al.* (1993). However, the structure of this compound was probably misinterpreted as far as the 3/5 substitution is concerned, which is a very uncommon substitution pattern (Westcott and Muir, 2000). It was more likely to be a ferulic acid glucoside (3-methoxy-4-O-β-D-glucopyranosyl-cinnamate), as supported by NMR analyses performed by Westcott and Muir (2000). Variations in phenolic acid content in flaxseed are largely attributable to seasonal effects (Oomah *et al.*, 1995). Flavonoids such as herbacetin diglucoside (HDG) and kaempferol diglucoside have also been identified in flaxseed (Qui *et al.*, 1999). The flavonoid content has been shown to be influenced by both cultivar and environment (Oomah *et al.*, 1996).

1.1.1 The lignan oligomer in flaxseed

In flaxseed, SDG does not exist as a free compound but is bound in a ester-linked oligomeric structure together with the hydroxycinnamic acid glucosides CoAG and FeAG (Bakke and Klosterman, 1956; Westcott and Muir, 2000; Struijs *et al.*, 2008), the flavonoid HDG (Struijs *et al.*, 2007), 3-hydroxy-3-methylglutaric acid (HMGA) (Klosterman and Smith, 1954; Ford *et al.*, 2001; Figure 2), and possibly other minor constituents.

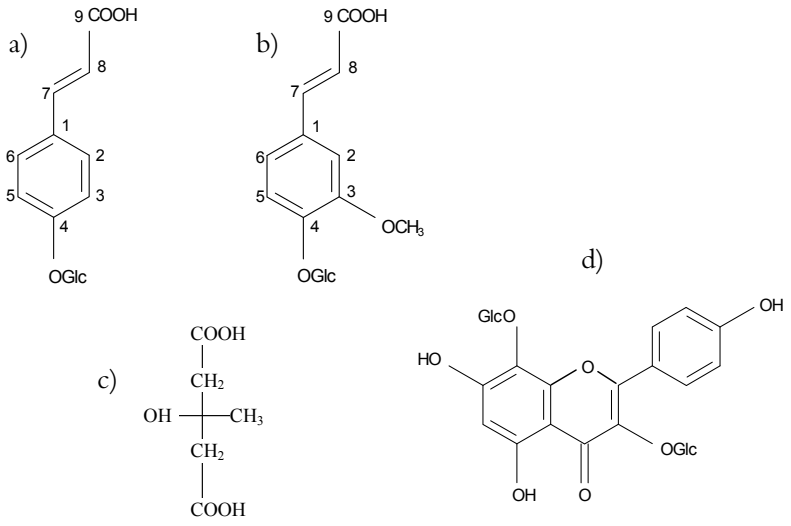

Figure 2. a) *p*-coumaric acid glucoside (MW=326 Da), b) ferulic acid glucoside (MW=356 Da), c) 3-hydroxy-3-methylglutaric acid (MW=162 Da), d) herbacetin diglucoside (MW=626 Da). For numbering of glucose carbons, see Figure 1b.

The ester linkages are mainly between the C-6 hydroxyl groups of the glucose units of SDG (Figure 1) and the carboxylic groups of CoAG, FeAG or HMGA (Ford *et al.*, 2001; Struijs *et al.*, 2008; Figure 2), although FeA, *i.e.* the aglycone of FeAG, has been shown to bind also to the C-2 position of glucose (Struijs *et al.*, 2008). Since HMGA possesses two carboxylic groups, it can bind to two glucose units from two separate SDG molecules, which means that the oligomer can elongate with SDG + HMGA in two directions. So far, there is only evidence of HMGA binding to one glucose residue in HDG, so it is still uncertain whether HDG is part of the backbone structure of the oligomer or whether it is a substituent (Struijs *et al.*, 2007). There is no evidence of CoAG and FeAG binding to constituents other than the glucose units in the oligomer (Struijs *et al.*, 2008). If CoAG or FeAG binds to the C-6 carbon in the glucose moiety of SDG or HDG it would serve as a terminal unit and thus block any further elongation of the oligomer in that direction. Struijs *et al.* (2009) therefore postulated that the size of the oligomer is determined by the level of inclusion of CoAG and FeAG. However, the molecular size distribution is wide, as shown by the single broad peak or 'hump' resulting from HPLC analyses of unhydrolysed extracts. As this peak is transformed to several sharp peaks upon alkaline hydrolysis (Westcott and Paton, 2001), its shape cannot be attributed to the chromatographic method. Instead, the broadness is the result of a wide mass distribution and/or differences in polarity between molecular species in the extract. A wide size distribution has also been shown by matrix-assisted laser desorption and ionisation time of flight mass spectroscopy (MALDI-TOF MS) performed on the unhydrolysed flaxseed extract (Struijs *et al.*, 2009).

Strandås *et al.* (2008a) fractionated the oligomeric extract according to polarity by solid phase extraction (SPE) by elution with 50, 60 and 70 % aqueous methanol and found that the 60% fraction was the largest fraction by mass and that its composition was most similar to that of the whole extract. It was also shown that when moving from 50% to 70% methanol, the relative content of SDG increased whereas the relative content of CoAG and FeAG decreased. Moreover, by separating the three fractions according to size, Strandås *et al.* (2008a) showed that the 50% fraction contained smaller molecules than the 60% and 70% fractions. Thus, Strandås *et al.* (2008a) provide some support for the hypothesis of Struijs *et al.* (2009) in that the 50% methanolic fraction, which is most abundant in CoAG and FeAG, seems to contain the smallest molecules.

1.1.2 Biosynthesis

Lignans, hydroxycinnamic acids and flavonoids originate from the phenylpropanoid pathway where the amino acid phenylalanine is the precursor (Davin and Lewis, 2003; Lepiniec *et al.*, 2006). The starting point of lignan biosynthesis is the deamination of phenylalanine by the enzyme phenylalanine ammonium lyase to yield cinnamic acid. Additional enzymatic steps yield *e.g. p*-coumaric acid, caffeic acid and ferulic acid. These phenolic acids can be converted into coenzyme-A activated forms which are reduced to aldehydes and finally alcohols, of which coniferyl alcohol is one example (Davin and Lewis, 2003). Two coniferyl alcohol radicals can be captured and coupled by a so-called dirigent protein to form the lignan pinoresinol (Davin *et al.*, 1997). The enzyme pinoresinol/lariciresinol reductase (Dinkova-Kostova *et al.*, 1996; Hano *et al.*, 2006) then mediates its conversion into first lariciresinol and then SECO, which can be further converted to matairesinol by the enzyme secoisolariciresinol dehydrogenase (Xia *et al.*, 2001). The glucosylation of SECO to form SDG has been suggested to be mediated by the enzyme secoisolariciresinol glucosyltransferase (Ford *et al.*, 2001) yet to be identified. Biosynthesis of the very diverse group of compounds that are collectively referred to as flavonoids starts with a reaction between coumaroyl-CoA deriving from the phenylpropanoid pathway and three malonoyl-CoA units deriving from the Krebs cycle (Lepiniec *et al.*, 2006).

By studying flaxseed at different developmental stages by stable and radioisotope precursor/tracer experiments, Ford *et al.* (2001) investigated the biosynthetic pathway of the flaxseed lignan oligomer. They concluded that the level of SDG in flaxseed increases during seed development and peaks at seed maturation. Since virtually no free SDG could be detected, it was assumed to be efficiently incorporated into the oligomeric structures once formed. CoAG and FeAG were found to be included into the oligomer at early stages of seed development. Coupling of SDG to HMGA was suggested to occur by coupling of glucose moieties with coenzyme A-activated HMGA.

1.1.3 Analysis of the phenolic compounds of the flaxseed lignan oligomer

Following the discovery of SDG by Bakke and Klosterman (1956) and its connection to the enterolignans, several methods for the analysis of lignans and other phenolic constituents of the fat free portion of flaxseed have been developed. In order to obtain repeatable results, defatting of flaxseed cake prior to further treatment is crucial (Kozlowska *et al.*, 1983; Harris and Haggerty, 1993).

Extraction of the oligomeric material is usually carried out with solvents such as dioxane/ethanol, aqueous ethanol or methanol (Bakke and Klosterman, 1956; Westcott and Muir, 1996a; Struijs *et al.*, 2009). Subcritical water extraction (Cacace and Mazza, 2006) and microwave-assisted extraction (Zhang and Xu, 2007) have also been employed. To release free compounds, mainly in the form of glucosides, the oligomeric extract can be treated with alkali (Rickard *et al.*, 1996; Westcott and Paton, 2001; Cacace and Mazza, 2006; Struijs *et al.*, 2009). The compounds may also be released from the oligomeric material as aglycones by enzymatic or acid hydrolysis (Obermeyer *et al.*, 1995; Mazur *et al.*, 1996; Liggins *et al.*, 2000; Charlet *et al.*, 2002). Without prior extraction of the oligomeric material, direct alkaline hydrolysis in water and microwave-assisted extraction with alkali have been used (Eliasson *et al.*, 2003; Beejmohun *et al.*, 2007).

Extraction for analysis of the other phenolic compounds such as phenolic acids and flavonoids has been carried out in a similar manner as when analysing lignans, *i.e.* with organic solvents (Bakke and Klosterman, 1956; Oomah *et al.*, 1995) or alcohols mixed with water (Kozlowska *et al.*, 1983; Amarowicz *et al.*, 1994; Oomah *et al.*, 1996; Westcott and Muir, 1996a; Ford *et al.*, 2001; Charlet *et al.*, 2002; Degenhardt *et al.*, 2002; Struijs *et al.*, 2009). The use of supercritical fluid (SCF) extraction has also been reported (Harris and Haggerty, 1993).

SDG, phenolic acids and flavonoids absorb light in the UV-region. They have been quantified and determined by liquid and gas chromatography and mass spectroscopic and NMR techniques (Bambagiotti-Alberti *et al.*, 1994; Mazur *et al.*, 1996; Westcott and Muir, 1996b; Chimichi *et al.*, 1999; Westcott *et al.*, 2000; Willför *et al.*, 2006; Struijs *et al.*, 2007). In quantifications, the differences in efficiency of different modes of extractions, hydrolyses and the use of different flaxseed raw material have resulted in a broad variation in yields. A selection of quantitative results for SECO is shown in Table 2.

Table 2. *Overview of some analytical methods for quantification of secoisolariciresinol (SECO) in flaxseed or defatted flaxseed meal (DFF)*[1]

SECO yield (mg/kg)	Extraction	Hydrolysis	Reference
0.4 in seeds (solvent extr.)[2]	1. Ethanol/dioxane 1:1 v/v	n.a.[3]	Harris and Haggerty, 1993
1400 in seeds (SCF extr.)[2]	2. Methanol		
	3. SCF CO_2		
820 in seeds	n.a.[3]	β-glucuronidase	Obermeyer *et al.*, 1995
2300 in DFF			
4700 in DFF[2]	Ethanol/dioxane 1:1 v/v	0.3 M sodium methoxide	Rickard *et al.*, 1996
3000-9600 in DFF[2]	70 % aqueous methanol	0.1 M NaOH	Westcott and Muir, 1996b
3700 in seeds	n.a.[3]	β-glucuronidase, acid	Mazur *et al.*, 1996
4200-12 600 in seeds	n.a.[3]	1.5 M HCl at 100 °C	Liggins *et al.*, 2000
6000 in seeds	70% aqueous methanol	2 M HCl at 100 °C	Charlet *et al.*, 2002
1200 in seeds[2]	70% aqueous methanol	1 M NaOH	Degenhardt *et al.*, 2002
6200-13 500 in seeds[2]	n.a.[3]	Direct hydr. with 2 M NaOH	Eliasson *et al.*, 2003
5300 in seeds[2]	Subcritical water extr.	1 M NaOH	Cacace and Mazza, 2006
5100 in pressed flaxseed cake[2]	Microwave-assisted extraction and hydrolysis in 70% aqueous methanol and 0.1 M NaOH		Beejmohun *et al.*, 2007

[1]The yield of DFF is ~50-60% of seed weight; [2] yielded the diglucoside of SECO (SDG). Values for SDG (MW=686) have been recalculated as SECO (MW=362); [3] n.a. = not applied

Due to varying methodologies, reports on quantities and types (*e.g.* free, ester-bound *etc.*) of phenolic acids in flaxseed are also diverging. Kozlowska *et al.* (1983) showed that the majority (59%) of phenolic acids in flaxseed were ester-bound. By extraction with 80% methanol, the content of ester-bound phenolic acids in flaxseed was determined to be 320 mg/kg defatted flaxseed flour (DFF), the main constituents being *p*-hydroxybenzoic, *trans*-ferulic and *trans-p*-coumaric acids. Free phenolic acids present at 220 mg/kg defatted flaxseed flour were mainly composed of *trans*- and *cis*-sinapic, *p*-hydroxybenzoic, *trans-p*-coumaric and vanillic acids. The content of residual (non-extractable with the method used) phenolic acids was 70 mg/kg DFF. Dabrowski and Sosulski (1984) found no free phenolic acids when extracting DFF with tetrahydrofuran. Alkaline hydrolysis released 730 mg phenolic acids/kg DFF (89% of total phenolic acids, the major forms being *trans*-ferulic and *trans*-sinapic acid). Harris and Haggerty (1993) reported a content of approximately 11 mg ferulic acid/kg in whole flaxseed. They did not use alkaline hydrolysis and thus could not detect ester-bound phenolic acids. Oomah *et al.* (1995) reported a content of 8000-10 000 mg total phenolic acids/kg flaxseed, of which 48-66% were assigned to esterified phenolic acids. Phenolic acids that were not released after extraction and alkaline hydrolysis were assumed to be ether-bound. Eliasson *et al.* (2003) reported a *p*-coumaric acid glucoside content of 1200-8500 mg/kg flaxseed (dry weight) and a ferulic acid glucoside content of 1600-5000 mg/kg using direct alkaline hydrolysis.

The mean total flavonoid content in flaxseed, measured by absorbance in 80% methanolic extract at 404 nm, has been reported to be 490-870 mg/kg DFF (Oomah *et al.*, 1996). Struijs *et al.* (2007) found 2000 mg/kg of HDG in flaxseed hulls, whereas Qui *et al.* (1999) only found 100 mg/kg in flaxseed.

1.1.4 Metabolism and physiological effects

In the early 1980s, the enterolignans enterodiol (ED) and enterolactone (EL) were identified as lignans of animal origin that differed from plant lignans in carrying phenolic hydroxyl groups only in the *meta* position of the aromatic rings (Figure 3). They were found in urine, plasma and bile as glucuronide and sulphate conjugates, and in unconjugated forms in faeces (Setchell *et al.*, 1979; Axelson and Setchell, 1980; Setchell *et al.*, 1980; Axelson and Setchell, 1981; Setchell *et al.*, 1981).

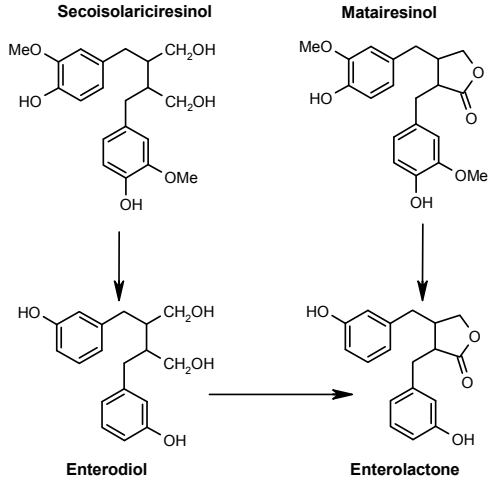

Figure 3. Conversion of mammalian lignan precursors SDG and matairesinol to enterodiol and enterolactone by the gut microflora (adapted from Borriello *et al.*, 1985).

Later, SDG, matairesinol (Axelson *et al.* 1982), lariciresinol, pinoresinol (Heinonen *et al.*, 2001) and lignin in plant cell walls (Begum *et al.*, 2004) were identified as enterolignan precursors. Fibre-rich foods such as cereals, oilseeds, fruits and vegetables are rich sources of these precursors (Adlercreutz *et al.*, 1982; Thompson *et al.*, 1991; Mazur, 1998; Milder *et al.*, 2005) (Table 1). SDG/SECO results in both EL and ED formation, with varying reports on which product is dominant (Axleson *et al.*, 1982; Saarinen *et al.*, 2002; Smeds *et al.*, 2004). Flaxseed supplementation has been reported to mainly increase ED concentration (Nesbitt *et al.*, 1999). ED and EL are formed by oxidation of precursors in a dose-dependent manner by facultative anaerobic bacteria in the colon of animals and humans (Axelson and Setchell, 1981; Setchell *et al.* 1981; Borrielo *et al.* 1985; Rickard *et al.*, 1996; Clavel *et al.,* 2006). Both the amount and the species composition of the bacteria seem to influence the production of enterolignans (Axelson and Setchell, 1981; Rowland *et al.*, 1999; Clavel *et al.*, 2005). The conversion has been shown to be enantiospecific (Saarinen *et al.*, 2002; Jin *et al.*, 2007a, b). Other factors that influence the bioavailability of enterolignans are precursor structure, intestinal transit time and time of exposure (Axelson *et al.*, 1982; Saarinen *et al.*, 2002; Smeds *et al.*, 2004). The composition and the structure of the food may also play a role. For example, enterolignan formation increased *in vitro* in xylanase-treated rye bran (Aura *et al.*, 2005).

The absorption of enterolignan precursors from flaxseed has been shown to increase when the seeds are crushed or milled (Kuijsten *et al.*, 2005). By *in vitro* fermentation with fresh human faecal inoculum, different flaxseed cultivars have been shown to produce different amounts of enterolignans (Thompson *et al.,* 1991), probably due to variations in precursor content between cultivars, but possibly also due to structural differences between the flaxseeds or the lignan oligomers. Chemically unchanged lignans (matairesinol, lariciresinol, isolariciresinol, secoisolariciresinol) have been detected in low amounts in human urine. The cause may be precursor overload or insufficient bacterial capacity (Bannwart *et al.*, 1989; Rickard *et al.*, 1996).

After absorption, ED and EL undergo enterohepatic circulation where glucuronic and sulphuric acid conjugates are formed in the liver, excreted in the bile, deconjugated by intestinal bacteria and then reabsorbed (Axelson and Setchell, 1980; Axelson and Setchell, 1981; Setchell *et al.*, 1981). Conjugated ED and EL are transported in plasma to tissues such as the kidneys and uterus (Axelson and Setchell, 1981; Rickard and Thompson, 1998). EL is normally the enterolignan found in the highest concentrations in blood and is also that most commonly examined in epidemiological studies (Setchell *et al.*, 1980b; Nesbitt *et al.*, 1999; Adlercreutz, 2007).

Enterolignans and their precursors are similar to steroids in size and polarity, and are sterically similar to 17β-oestradiol (Figure 4) regarding the distance between the two -OH groups (Setchell *et al.*, 1981; Tham *et al.*, 1998). Indeed, they have been shown to interact with oestrogen metabolism at different levels. In circulation, enterolignans and precursors compete with endogenous hormones in binding to sex hormone-binding globulin (SHBG) (Martin *et al.*, 1996; Schöttner *et al.*, 1997; Schöttner *et al.*, 1998; Hillerns *et al.*, 2005) and influence its biosynthesis (Adlercreutz *et al.*, 1987). Enterolignans have also been shown to interfere with oestradiol biosynthesis by decreasing the activity of aromatase and 17β-hydroxysteroid dehydrogenase (Wang *et al.,* 1994; Brooks and Thompson, 2005), which are the enzymes responsible for final conversion of steroid precursors to oestradiol. In addition, the enterolignans affect the activity of oestrogen receptors as observed as displacement of endogenous oestrogen, effects on gene transcription and cell proliferation (Mueller *et al.*, 2004; Cosentino *et al.*, 2007; Carreau *et al.*, 2008) and *in vivo* effects on target tissues (Setchell *et al.*, 1981; Waters and Knowler, 1982; Penttinen *et al.*, 2007). Decreased formation and circulation of endogenous oestrogens limit stimulation of oestrogen-dependent tissues. A compound with weak oestrogenic effect can be anti-oestrogenic when competing with a compound that has a stronger

oestrogenic effect. The effect is dependent on the dose, timing and duration of exposure (Tou *et al.*, 1999). In a study by Mousavi and Adlercreutz (1992), oestradiol and enterolactone individually stimulated the growth of MCF-7 breast cancer cells *in vitro*, but together they inhibited each other's effect.

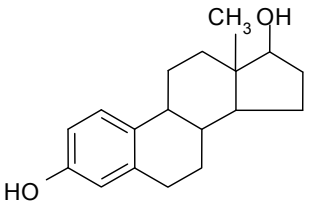

Figure 4. Structure of 17β-oestradiol.

The flaxseed lignans SDG/SECO and to some extent the enterolignans have been shown to possess antioxidant activities (Prasad, 1997b, 2000a; Kitts *et al.*, 1999; Niemeyer and Metzler, 2003; Eklund *et al.*, 2005; Hosseinian *et al.*, 2006, 2007), which may protect body lipids from peroxidation. SDG has a lower antioxidant capacity than SECO (Hosseinian *et al.*, 2007).

Only one report on possible adverse health effects of flaxseed lignans could be found in the literature (Jacobs *et al.*, 2005). EL and SECO were shown to activate the human pregnane X receptor (PXR), which mediates the induction of enzymes involved in steroid metabolism and xenobiotic detoxification. The authors suggest that this activation may possibly interfere with the action of the vast amount of pharmaceutical drugs that are cytochrome P4503A substrates.

1.1.5 Health effects of mammalian and flaxseed lignans

Breast cancer

A reduced breast cancer risk has been reported for subjects with high urine and plasma levels of enterolignans (Ingram *et al.*, 1997; Dai *et al.*, 2002; Boccardo *et al.*, 2004). Blood concentrations of EL above 16 nmol/L were associated with a reduced risk of breast cancer in a study with 366 cases and 733 matched controls (Sonnestedt *et al.*, 2008b). However, in several other studies no association between plasma enterolignan levels and a reduced risk of breast cancer could be established (den Tonkelaar *et al.*, 2001; Kilkkinen *et al.*, 2004; Ward *et al.*, 2008). Recently, three meta-analyses on the relationship between breast cancer risk and plant lignan intake, enterolignan

exposure and blood EL levels (Velentziz *et al.*, 2009) showed little association between high plant lignan intake and a reduced cancer risk overall, but when the analysis was restricted to post-menopausal women a clear relationship was obtained. High enterolignan exposure was associated with a reduced breast cancer risk but blood EL levels were not. Conflicting results in human studies may depend on different biological properties of different tumour types or on too low concentrations of enterolignans in the populations studied (Sonnestedt *et al.*, 2008b).

Different results from studies on the protective effects of flaxseed lignans have also been reported. In an intervention study performed by Thompson *et al.* (2005), a daily intake of flaxseed for a month by 19 newly diagnosed postmenopausal breast cancer patients indicated protective effects of flaxseed lignans. Pure SDG when administered to rats at the early promotion stage of mammary tumourigenesis was shown to reduce the number of tumours. At the same time, urinary excretion of enterolignans was shown to increase significantly (Thompson *et al.*, 1996b). The total volume of established and new mammary tumours was significantly decreased in rats that were fed SDG or flax. The effect was inversely related to urinary excretion of enterolignans (Thompson *et al.*, 1996a). SDG also resulted in a reduction in the progression of mammary tumourigenesis in rats (Rickard *et al.*, 1999), and pulmonary metastasis (Li *et al.*, 1999) and distant metastasis, but not recurrence after surgical removal of tumours in mice (Chen *et al.*, 2006).

Apart from hormonal effects, the antioxidant properties of lignans have also been suggested to play a role in cancer protection (Prasad, 2000a). Protective effects of flaxseed against breast cancer that may not be attributed to lignans alone have also been observed. When SDG and flaxseed oil were administered together, they showed a synergistic protective effect (Chen *et al.*, 2006), possibly related in part to the alpha-linolenic acid in the oil (Thiébaut *et al.*, 2009).

A high-fibre diet with its associated lignans seems to account for the cancer protective effects (Adlercreutz, 2007; Sonnestedt *et al.*, 2008a) but the exact role of lignans is still unclear. The main theory seems to be that the lignans modulate the activity of endogenous oestrogen but it is possible that *e.g.* enterolignans are only markers for a healthy diet and that the true underlying mechanisms have not yet been discovered (Adlercreutz, 2007; Saarinen *et al.*, 2007).

Prostate cancer

High levels of enterolactone in prostatic fluid have been suggested to indicate a lower risk of prostate cancer (Morton *et al.*, 1997). A reduction in

prostate tumour proliferation, prostate-specific antigen and total and free testosterone levels has been observed in patients with prostate cancer given a low-fat diet containing flaxseed (Demark-Wahnefried *et al.*, 2001, 2004, 2008).

Inclusion of 5% flaxseed in the diet inhibited growth and development of prostatic carcinoma in mice, possibly due to hormonal effects of the flaxseed lignans (Lin *et al.*, 2002). In male rats with experimental cancer, a 5% lifetime diet reduced prostate cell proliferation rate and prostate weight without a rise in sex hormone levels. A 10% lifetime diet, on the other hand, resulted in higher serum testosterone and oestradiol levels and prostate cell proliferation (Tou *et al.*, 1999). EL and ED may also contribute to the protective effects, as indicated by an *in vitro* study where these lignans reduced the growth of three and two different prostate cancer cell lines, respectively (Lin *et al.*, 2001).

Colorectal cancer

Dietary lignans have also been suggested to protect against colon cancer (Adlercreutz, 2002). However, studies on the effect of flaxseed lignans against colorectal cancer are sparse. Diets supplemented with flaxseed, flax meal or SDG showed a protective effect against colon cancer in rats in the long term (Jenab and Thompson, 1996). A reduction in early markers for colorectal cancer in rats fed flaxseed flour or flaxseed meal was also shown. The reduction was not linearly correlated to flaxseed intake (Serraino and Thompson, 1992). Induced colorectal cancer in multiple intestinal neoplasia (Min) mice was significantly reduced by flaxseed supplemented diets (Bommareddy *et al.*, 2009). However, in other studies no effects of flaxseed or (-)-SECO were obtained in Min mice models (van Kranen *et al.*, 2003; Oikarinen *et al.*, 2005; Pajari *et al.*, 2006).

Cardiovascular disease

Cardiovascular disease is a collective term for coronary heart disease (heart attacks), cerebrovascular disease, elevated blood pressure (hypertension), peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure (WHO, 2009a). Important risk factors for developing cardiovascular disease include diabetes, hypertension, smoking, obesity, high plasma cholesterol (hypercholesterolaemia), lack of physical activity and heredity (WHO, 2009b). Cardioprotective effects of flaxseed or enterolignans have been observed in humans and animals and may be due to inhibition of lipid peroxidation or changes in SHBG production, oestrogenic activity and circulation of cholesterol (Fahrat *et al.*, 1996;

Vanharanta *et al.*, 1999; Chisolm and Steinberg, 2000; Vanharanta *et al.*, 2002; Lucas *et al.*, 2004).

High serum enterolactone concentrations have been correlated with a decreased level of *in vivo* lipid peroxidation (Vanharanta *et al.*, 2002), and are also protective against acute coronary heart events in men as suggested by a case-control study (Vanharanta *et al.*, 1999). In a prospective cohort study, men with elevated serum EL levels had a reduced risk of coronary heart or cardiovascular disease-related mortality (Vanharanta *et al.*, 2003). The lignan phenolic complex did not affect plasma lipid concentrations, antioxidant capacity or endothelial function in healthy postmenopausal women (Hallund *et al.*, 2006a, b). In hypercholesterolaemic subjects, however, a daily intake of the complex reduced plasma LDL and cholesterol levels by more than 20% (Zhang *et al.*, 2008). Flaxseed supplementation had a modest effect on plasma cholesterol levels in healthy menopausal women (Dodin *et al.*, 2008).

The phenolic complex, SDG and flaxseed low in alpha-linolenic acid (<3%) were shown to decrease the level of atherosclerotic lesions and plaques in hypercholesterolaemic rabbits (Prasad *et al.,* 1998; Prasad, 1999, 2005). Most experimental animal studies have shown a reduction in plasma cholesterol levels following consumption of flaxseed or SDG (Prasad *et al.,* 1998; Prasad, 1999, 2005; Lucas *et al.*, 2004; Pellizzon *et al.*, 2007). However, in a study by Prasad (1997a), the cholesterol levels in rabbits increased after consumption of flaxseed. Furthermore, SDG and its oligomer, when fed to rats, were shown to significantly increase the amount of cholesterol in the liver and liver lipids and to decrease the liver and plasma content of α- and γ-tocopherols in rats (Frank *et al.*, 2004). Moreover, flaxseed was found to cause a significant reduction in α- and γ-tocopherol levels in rats (Ratnayake *et al.*, 1992). Reduced levels of vitamin E, γ-tocopherol in particular, have been associated with an increased risk of cardiovascular disease (Hensley *et al.*, 2004). Other effects on lipid metabolism were shown in a study by Fukumitsu *et al.* (2008), where SDG and ED had beneficial effects on lipid metabolism in diet-induced fat accumulation in mice. Their findings suggest that the compounds regulate adipogenesis-related gene expressions by increasing the PPARγ DNA-binding activity. PPARγ is a transcription factor (*i.e.* a protein that binds to specific DNA sequences and controls transcription) that is involved in regulation of adipogenesis, lipid and glucose metabolism (Rosen *et al.*, 2000)

Diabetes

Diabetes is a chronic disease caused by deficiency in insulin production or by ineffectiveness of the insulin produced, resulting in increased blood

glucose levels which cause damage to body systems, in particular the blood vessels and nerves (WHO, 2009c).

Hypercholesterolaemic postmenopausal women showed reduced glucose and insulin levels after eating flaxseed (Lemay *et al.*, 2002). In another group of hypercholesterolaemic subjects, the phenolic complex reduced the fasting plasma glucose levels (Zhang *et al.*, 2008). In a study by Pan *et al.* (2007), hypercholesterolaemic postmenopausal women diagnosed with type II diabetes showed small improvements in long-term glycaemic control but no effect on fasting glucose or insulin sensitivity after eating low amounts of the phenolic complex for eight weeks. SDG has been demonstrated to prevent or delay the development of type I and II diabetes in diabetes-prone rats, probably by decreasing oxidative stress in body tissues (Prasad, 2000b, 2001).

1.2 Flaxseed oil

Among dietary plant oils, flaxseed oil has one of the highest contents of the essential omega-3 fatty acid alpha-linolenic acid (ALA), with amounts of 50-60% (Velasco and Goffman, 2000; Hall *et al.*, 2009). The second and third most dominant fatty acids in flaxseed oil are oleic acid and the essential omega-6 fatty acid linoleic acid (LA), with 11-29 and 9-21% respectively (Green and Marshall, 1981; Velasco and Goffman, 2000; Wakjira *et al.*, 2004; Choo *et al.*, 2007). The oil content in the seed is in the range 22-47% (Velasco and Goffman, 2000; Eliasson *et al.*, 2003; Wakjira *et al.*, 2004). Both oil content and fatty acid composition depend on factors such as cultivar, location, environmental conditions and agricultural practices, the importance of which have been reviewed by Oomah and Kenaschuk (1995). Flaxseed lipids consist of more than 90% triacylglycerols, with the remaining part being free fatty acids, phospholipids, sterols and sterol esters (El Shattory, 1976).

The unsaponifiable portion of flaxseed oil, mainly consisting of sterols and tocopherols, comprises about 0.4-1.4% of total lipids (Painter and Nesbitt, 1943; Choo *et al.*, 2007). The main sterols are sitosterol, cycloartenol and campesterol, together making up 75% of total sterols (Schwartz *et al.*, 2008). The total tocopherol content in flaxseed is 9.3-16.9 mg/100 g seed (Oomah *et al.*, 1997; Velasco and Goffman, 2000), with the major tocopherol being γ-tocopherol, which makes up 95-100% of total tocopherols (Oomah *et al.*, 1997; Velasco and Goffman, 2000) and is present at levels of 11.2-52.0 mg/100 g oil (Choo *et al.*, 2007; Schwartz *et al.*, 2008). Other tocopherols are α-tocopherol, with a content of 0.0-9.1 mg/100 g oil (Choo *et al.*, 2007; Schwartz *et al.*, 2008) and δ-tocopherol at

0.0-0.3 mg/100 g seed (Oomah *et al.*, 1997; Velasco and Goffman, 2000) or 0.95 mg/100 g oil (Schwartz *et al.*, 2008). The tocopherol content is mainly influenced by cultivar but also by location and year (Oomah *et al.*, 1997). Other minor constituents that have been identified in flaxseed oil include the tocopherol analogue plastochromanol-8 (Velasco and Goffman, 2000), the carotenoids β-carotene, lutein and violaxanthin (Pretova and Vojtekova, 1985), squalene and aliphatic hydrocarbons (Herchi *et al.*, 2009) and geranyl geraniol (Fedeli *et al.*, 1966). Flaxseed oil has also been found to contain 768-3073 mg/kg of total phenolic acids and 127-256 mg/kg of total flavonoids (Choo *et al.*, 2007).

Apart from agroclimatic conditions, the composition of vegetable oils also depends on the extraction procedure and the treatment of the oil. A refined oil is the result of treating a crude oil by a multiple-step procedure often including *e.g.* filtration, degumming, neutralisation, bleaching, deodorisation and winterisation (Karlshamns AB, 1989). These procedures rid the oil of *e.g.* solid particles, phospholipids, proteins, carbohydrates, free fatty acids, waxes and compounds giving unwanted taste and odour and also prolong the shelf-life of the oil. However, the procedures may also decrease the amount of desirable minor compounds present (Kamal-Eldin, 2005). The so-called virgin or cold-pressed oils are extracted under milder conditions, purified by filtration or settling and not subjected to other refining steps (Moreau and Kamal-Eldin, 2009).

Minor compounds of vegetable oils, especially tocopherols, phytosterols and phenolic compounds such as those found in olive oil and the lignans in sesame oil, have attracted increased attention lately due to their biological and physiological effects and their potential ability to improve human health (Kamal-Eldin, 2005).

1.2.1 Significance of omega-3 fatty acids for health

The omega-3 (n-3) fatty acid ALA (18:3 n-3) and the omega-6 (n-6) fatty acid LA (18:2 n-6) (Figure 5) are essential fatty acids in that they are needed to maintain health (Burr and Burr, 1930) but cannot be synthesised from any dietary precursors by humans so they have to be present in the diet. As components of cell membranes, n-3 and n-6 fatty acids increase membrane fluidity and play an important role for the function of cell membranes and the nervous system (Davis and Kris-Etherton, 2003). When naming fatty acids, the number to the left of the colon signifies the number of carbon atoms and the number to the right the degree of unsaturation, *i.e.* the number of double bonds in the carbon chain. The omega-3 and omega-6 (or n-3 and n-6) numbers describe the position of the first double bond

counting from the methyl end of the chain. ALA and LA can be elongated (*i.e.* chain length increases) and desaturated (*i.e.* the number of double bonds increases) to longer-chain n-3 and n-6 fatty acids, respectively, by a series of enzymatic reactions that take place in the endoplasmic reticulum and the peroxisomes (Figure 6). Double bonds are inserted by enzymes called desaturases. The position of insertion is counted from the carboxyl end of the chain and is given a delta number (Δ= delta). Higher animals lack the ability to insert double bonds beyond Δ9. Chain length is increased by enzymes called elongases. The most important products of these elongation and desaturation reactions are the highly unsaturated fatty acids (HUFA; chain length ≥20 carbons and ≥3 double bonds) eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (AA).

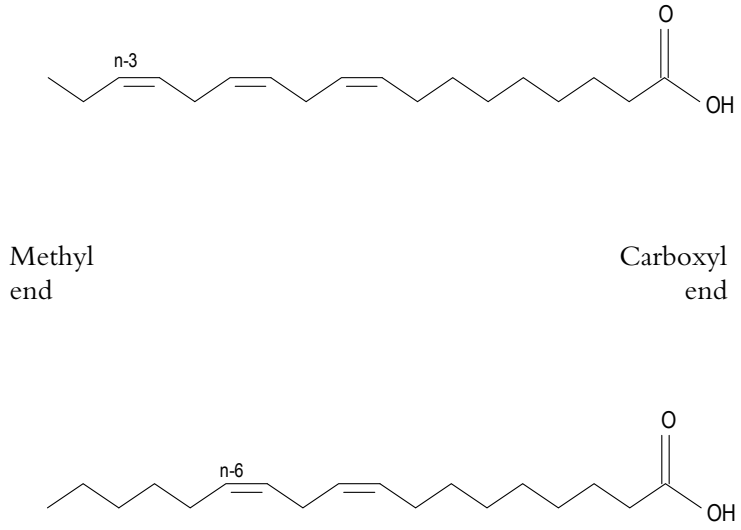

Figure 5. Chemical structure of the essential fatty acids alpha-linolenic acid (18:3 n-3; above), and linoleic acid (18:2 n-6; below).

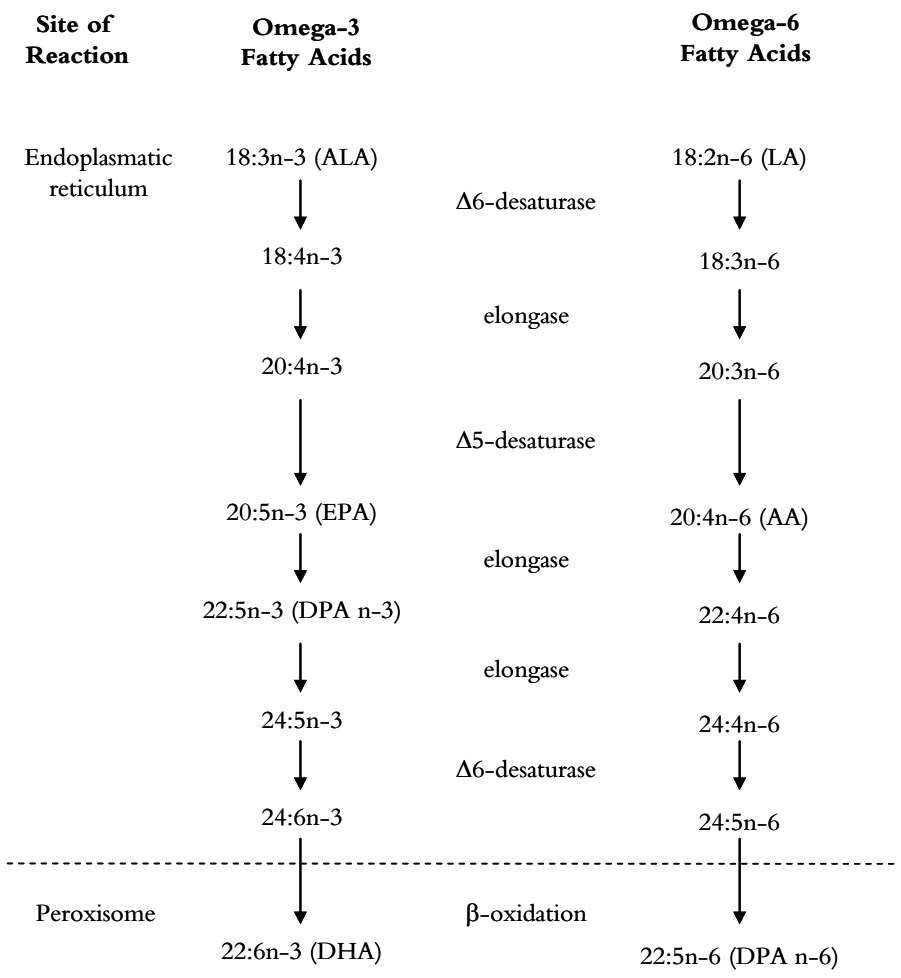

Figure 6. The metabolic pathway of omega-3 (n-3) and omega-6 (n-6) fatty acids. Adapted from Voss *et al.*, 1991.

EPA and AA are substrates for the production of hormone-like compounds called eicosanoids. The eicosanoids from EPA have weak activity and inhibit inflammation, while eicosanoids from AA have strong activity that promotes inflammation (Harper and Jacobson, 2001). Excessive action of eicosanoids from the n-6 fatty acid AA is associated with health disorders such as coronary heart disease (Lands, 2001). On the other hand, there is strong epidemiological evidence that consumption of oils rich in n-3 fatty acids such as ALA, EPA and DHA lowers the incidence of coronary heart disease (Dyerberg *et al.*, 1978; de Lorgeril *et al.*, 1994; Lanzmann-Petithory, 2001; Harper and Jacobson, 2001).

At present, the Swedish National Food Administration recommends an intake of 5-10% energy of polyunsaturated fatty acids per day, of which 1 %-unit should be n-3 fatty acids (Livsmedelsverket, 2009). Not only a specific dietary intake of n-3 fatty acids but also the balance between n-3 and n-6 fatty acids may be important (Lanzmann-Petithory, 2001; Simopoulos, 2008). The elongation and desaturation of ALA and LA involve the same enzymes (Figure 6), so the intake of LA may affect the conversion of ALA into longer-chain metabolites and *vice versa* (Simopoulos, 2008). In an evolutionary perspective, the n-6 fatty acids in our diet have increased recently due to extensive use of vegetable oils with very high LA/ALA ratios in food manufacturing and also because farm animals are fed diets high in LA, leading to meat that is 'enriched' in n-6 fatty acids (Simopoulos, 1991; Wood and Enser, 1997; Nilzen *et al.*, 2001; Eriksson and Pickova, 2007).

Dietary sources with high ALA/LA ratios include flaxseed, perilla and camelina oil, and walnuts (Harper and Jacobson, 2001; Hall *et al.*, 2009). Apart from being formed in the body by elongation and desaturation of ALA, n-3 HUFA can also be ingested through food. Fish is an important source of n-3 HUFA.

1.2.2 Flaxseed oil as an ingredient in fish feed

Long chain n-3 fatty acids such as EPA, DPA and DHA are readily available in fish oil, which is a common lipid ingredient in commercial fish feed. Lipids in tissues reflect the lipid content in the diet (Sargent, 2002) and the presence of n-3 HUFA in feed also results in fish flesh with high relative amounts of these fatty acids, which are beneficial for human consumers. However, fish oil resources for fish feed are becoming scarce, so alternative lipid sources must be found (FAO, 2002). For this reason, partial or

complete substitution of fish oil with vegetable oils in fish feed is being assessed.

Salmonids, *e.g.* Atlantic salmon (*Salmo salar*), have some ability to convert ALA and LA, present in high concentrations in many vegetable oils, to long-chain HUFA (Sargent, 2002). Flaxseed oil contains high levels of n-3 desaturation/elongation precursors in the form of ALA, so it should be a good candidate for fish oil substitution in fish feeds (Tocher *et al.*, 2002). Numerous studies on Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) have shown that feed containing flaxseed oil does not have any negative effects on fish growth (Bell *et al.*, 1997, 2003, 2004; Tocher *et al.*, 2000; Menoyo *et al.*, 2005). However, despite observed increased desaturation and elongation activities (Bell *et al.*, 1997; Tocher *et al.*, 2002), flaxseed oil diets and other vegetable oil diets have failed to maintain the n-3 HUFA levels in fish tissues (Thomassen and Røsjø, 1989; Tocher *et al.*, 2000; Bell *et al.*, 2003; Menoyo *et al.*, 2005; Chen *et al.*, 2006).

The sesame seed lignan sesamin (Figure 1c) has been shown to increase the relative amount of DHA in white muscle of rainbow trout fed a diet with fish oil completely replaced with vegetable oils (Trattner *et al.*, 2008). A combination of flaxseed oil and sesamin led to lower production of DHA than a 6:4 (v/v) mixture of flaxseed oil and sunflower oil and sesamin in rainbow trout. The fact that sesamin added to the mixed oil resulted in higher levels of DHA compared with sesamin added to flaxseed oil was surprising, since flaxseed oil contains more ALA than the mixed oil and thus more substrate for DHA formation. The negative effect of flaxseed oil on n-3 HUFA formation has been proposed to be caused by the high ALA/LA ratio in flaxseed oil (Tocher *et al.*, 2002). However, the effect might also be attributable to minor components present in the flaxseed oil (Kamal-Eldin, 2005) possibly interfering with the desaturation and elongation of ALA or with other steps in lipid uptake and metabolism.

Even though flaxseed oil has so far not been proven to maintain the levels of n-3 HUFA in fish flesh, it may still have an advantage over other vegetable oils in that it results in fish flesh with a high ALA content and thus a high total n-3 fatty acid content (Menoyo *et al.*, 2005; Chen *et al.*, 2006). The effects of different diets on the lipid composition of fish may differ in different fish species, in fish at different developmental stages and in anadromous fish such as salmon and trout due to *e.g.* fresh/salt water transfer (Bell *et al.*, 1997; Tocher *et al.*, 2000; Sargent, 2002; Zheng *et al.*, 2005). There is also a large individual variation in the ability of fish to maintain high levels of n-3 HUFA in their flesh (Schlechtriem *et al.*, 2007).

2 Objectives

The specific aims of this thesis were:

To develop an HPLC method for the quantification of SDG in flaxseed (Paper I).

To identify two major peaks that elute before SDG in the HPLC chromatograms from the method developed in Paper I, and to obtain preliminary information on the flaxseed lignan oligomer by chromatographic and spectroscopic analyses (Paper II).

To elucidate the structure of the flaxseed lignan oligomer by chromatographic and spectroscopic analyses (Paper III).

To test the hypothesis that higher SDG to hydroxycinnamic acid ratio in flaxseed is associated with smaller lignan oligomer size (Paper IV).

To test the effect of purified flaxseed triacylglycerols and sesamin on the fatty acid composition in white muscle of rainbow trout (Paper V).

3 Analytical procedures

3.1 Work related to the lignan oligomer of flaxseed (Papers I-IV)

An outline of the work resulting in Papers I-III is shown in Figure 7. For more detailed descriptions of the procedures presented below, see Papers I-IV.

In Paper I, SDG was purified from a flaxseed extract by reversed and normal phase column chromatography for use as an external standard in quantifications and for future reference. The identity and purity of the isolated SDG were determined by proton nuclear magnetic resonance (^{1}H NMR). A high performance liquid chromatography (HPLC) method for the quantitative determination of SDG in flaxseed was developed and used for analysis of SDG in Swedish (n = 14) and Danish (n = 15) flaxseed cultivars. SDG was detected by ultra violet-diode array detection (UV-DAD) and its content in flaxseed was calculated with the help of a standard curve produced using the purified SDG at the concentrations 0, 20, 40, 80, 120 and 160 μg/mL ($y = 3.69x - 9.21$; $R^2 = 0.999$).

In Paper II, an unhydrolysed oligomeric flaxseed extract (referred to in the paper as the whole polymer) was fractionated with 0-100% methanol in 10 %-unit intervals on a C18 reversed phase solid phase extraction (SPE) column yielding 11 fractions. These fractions were analysed by HPLC. The main UV-absorbing fractions, shown to be eluted with 50, 60 and 70 % aqueous methanol, and the whole extract were analysed by one- and two-dimensional NMR techniques (^{1}H NMR, COSY, HSQC-DEPT and HMBC) to obtain structural information on the oligomer. The same oligomeric fractions were also subjected to alkaline hydrolysis and analysed by HPLC.

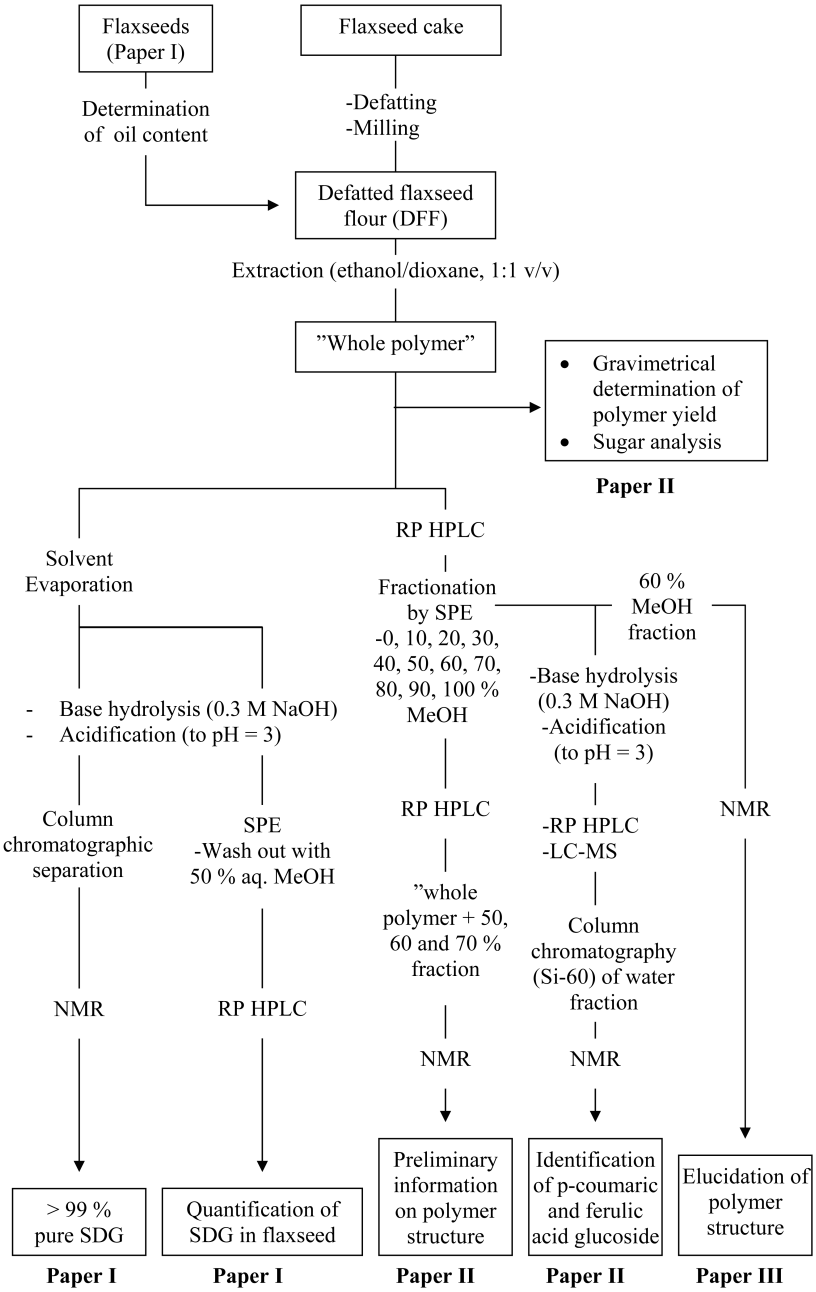

Figure 7. Overview of analytical procedures and outcomes of Papers I-III.

SDG was identified by comparison with a standard (Paper I) and preliminary information on the two other major peaks in the chromatograms was obtained by LC-MS and NMR. The compounds representing the two HPLC-peaks were isolated from an aqueous SPE fraction by normal phase column chromatography and identified by NMR. The sugar content and the yield of the whole extract were determined by qualitative sugar analysis (Theander *et al.*, 1995) and gravimetrical determination, respectively.

In Paper III, the major fraction of the oligomeric extract was obtained by washing the whole extract on a SPE column with 50% aqueous methanol followed by elution with 60% methanol. To elucidate the structure of the SDG-complex, the fraction was analysed by different NMR techniques (^{1}H NMR, ^{13}C NMR, TOCSY, HSQC-DEPT and HMBC). Standard SDG (see above) and HMGA were used as reference compounds.

In Paper IV, further insights were sought into the properties of the lignan oligomer. Seeds from seven flax cultivars (Agraf 17, Biltstar, Early Bird, Niagara, Talon, Taurus, Vimy) with different ratios of SDG to CoAG and FeAG (Westcott and Paton, 2001; Eliasson *et al.*, 2003) were used. Oligomeric extracts were obtained by extracting DFF according to Struijs *et al.* (2007) and the extracts were purified by reversed phase C18 SPE. The yields of the oligomeric extracts were determined gravimetrically. The extracts were analysed by reversed phase ultra-high performance liquid chromatography (UHPLC) and gel permeation chromatography (GPC) using the same columns and conditions as in Struijs *et al.* (2007).

For quantification of CoAG, FeAG, HDG and SDG, the oligomeric extracts were subjected to alkaline hydrolysis with 75 mM sodium hydroxide according to Struijs *et al.* (2009). Hydrolysed samples were purified by SPE. Samples were analysed by UHPLC-MS and chromatographic peaks were assigned by comparison with their mass spectra. The amounts of CoAG, FeAG, HDG and (+)-SDG and (-)-SDG in the extracts were determined with reference to standards.

Finally, fractions of the unhydrolysed lignan oligomeric extracts eluted from SPE columns with 50, 60 and 70 % aq. methanol were collected. The percentage contribution of each fraction to their combined yield was determined gravimetrically. Combined fractions were analysed by matrix assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS).

3.2 Work related to flaxseed oil (Paper V)

A feeding trial was conducted in which rainbow trout (*Oncorhynchus mykiss*) were fed six experimental diets containing purified flaxseed triacylglycerols, unaltered flaxseed oil or a mixed oil (sunflower oil/flaxseed oil), with and without the addition of the sesame lignan sesamin. The analytical procedures undertaken are summarised below. For further details see Paper V.

3.2.1 Feed preparation

By essentially following the method of Fuster *et al.* (1998), a commercial refined food-grade flaxseed oil was purified by adsorption chromatography, to obtain a purified flaxseed oil (PFSO) that was composed mainly of triacylglycerols (TAGs). Different batches of unaltered flaxseed oil (FSO) were mixed in order to obtain a α-linolenic (ALA) and linoleic acid (LA) composition that was comparable to that in the PFSO. (+)-γ-tocopherol and (+)-α-tocopherol were added to the PFSO and the FSO to make their tocopherol levels comparable. Among other ingredients, diets were prepared to contain 22% defatted fish meal and 22% vegetable oil. Sesamin (98% purity) was added at 0.58 g 100^{-1} g feed according to Trattner *et al.* (2008). The six diets used were: Flaxseed oil (FSO), flaxseed oil with added sesamin (FSO+S), purified flaxseed oil (PFSO), purified flaxseed oil with sesamin (PFSO+S), flaxseed and sunflower oil mixed 6:4 v/v (MO), and the mixed oil with sesamin (MO+S).

3.2.2 Fish

A total of 114 juvenile rainbow trout from northern Sweden were divided into six groups and each group was fed one of the six experimental diets (see above) *ad libitum* for 58 days. At harvest, the fish were dissected and then kept at -80 °C until further analysis. Only fish that had eaten the feed and that had at least doubled their weight were analysed for fatty acid composition and tocopherol and sesamin levels.

3.2.3 Lipid extraction and determination of fatty acids

Lipids from white muscle and diets were extracted following the method of Hara and Radin (1978). For fatty acid analysis, total lipids of fish white muscle were separated into TAG and PL according to Pickova *et al.* (1997). The total lipids from the diets and TAG and PL from white muscle were methylated according to Appelqvist (1968) and analysed by GC using the same column and conditions as in Trattner *et al.* (2008).

3.2.4 Sesamin and tocopherol determinations

For the analysis of sesamin and α- and γ-tocopherols in oils, feed and fish white muscle, lipids were dissolved in hexane and analysed by HPLC using the same system, column and conditions as described by Moazzami and Kamal-Eldin (2006). The concentrations of α- and γ-tocopherol and sesamin were determined by reference to authentic standards.

4 Results and discussion

4.1 Work related to the lignan oligomer of flaxseed (Papers I-IV)

In 1998, at the beginning of this work, the nature of the extractable phenolic polymeric material first described by Klosterman and Smith (1954) was not known. Moreover, reports on the SDG/SECO content in flaxseed showed great variation (Table 2), and values were often surprisingly low considering the high amounts of mammalian lignans excreted in urine of humans and animals after flaxseed ingestion (Axelson *et al.*, 1982; Thompson *et al.*, 1991). Our aim was thus to develop a quantitative method for the analysis of SDG in flaxseed and to learn more about the 'complex' in which SDG is included. This work is described in detail in Papers I-IV (see Appendix) and is summarised below.

4.1.1 Method development

In Paper I, defatted flaxseed flour (DFF) was extracted with 1,4-dioxane/95% ethanol (1:1, v/v) and hydrolysed in methanolic base according to Bakke and Klosterman (1956). Upon repeated HPLC analyses, it was discovered that the first two peaks (later assigned to *p*-coumaric acid and ferulic acid glucosides, see below) were diminished and some extra peaks eluted later, close to the SDG peak. When hydrolysis was carried out with aqueous base instead, the two peaks remained stable, suggesting formation of less polar derivatives due to transesterification of carboxylic groups in the methanolic system. The yield of SDG was unaffected by the method of hydrolysis, but the separation of HPLC peaks around the SDG peak was better when hydrolysis was performed in water. Thus, aqueous base hydrolysis was chosen for further work.

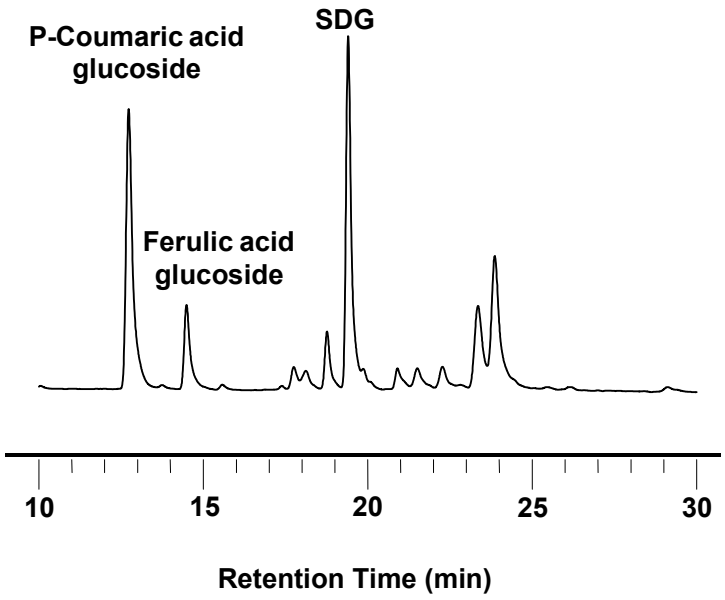

Figure 8. Typical chromatogram of a base-hydrolysed extract from flaxseed.

Figure 8 presents a typical HPLC chromatogram of the base-hydrolysed extract from flaxseed. The HPLC gradient developed in this study provided very good separation for SDG and easier interpretation of its UV-DAD and LC-MS spectra.

The method developed was used to study the variation in SDG content in flaxseed cultivars grown in Sweden (n = 14) and Denmark (n = 15). The level of SDG in these 29 samples varied between 11700 and 24100 mg/kg in DFF and between 6100 and 13300 mg/kg in whole seeds. These levels are in agreement with those reported by Rickard *et al.* (1996), Westcott and Muir (1996b), Liggins *et al.* (2000) and Charlet *et al.* (2002) (Table 2; yields reported as SECO). Eliasson *et al.* (2003) analysed 27 different cultivars with direct alkaline hydrolysis and reported an approximately two-fold higher mean SDG yield than was observed using the method developed in the present study (Table 2). The difference may be due to the efficiency of the analytical procedure, but may also be due to differences between years, cultivars or growing locations. Westcott and Muir (1996b) found a two-fold variation in flaxseed lignan concentration to be mainly due to cultivation year, with flax variety of secondary importance and cultivation location of less importance.

4.1.2 Isolation and structural determination of *p*-coumaric and ferulic acid glucoside

In Paper II, LC-MS of the first two peaks in the chromatograms of the hydrolysed oligomeric extract from the HPLC method developed (see above) (at 12.8 and 14.6 min; Figure 8) showed ions corresponding to 4-O-β-D-glucopyranosyl-*p*-coumaric acid and 4-O-β-D-glucopyranosyl-ferulic acid. The structures of these compounds were determined by NMR and were found to be in accordance with NMR data presented by Luyengi *et al.* (1993) and Westcott *et al.* (2000) for 4-O-β-D-glucopyranosyl-*p*-coumaric acid and 4-O-β-D-glucopyranosyl-ferulic acid, respectively.

4.1.3 Structural analyses of the lignan oligomer

Paper II also sought insights into the structure of the flaxseed oligomer (called polymer in that paper). The yield of the oligomeric extract from DFF was 2.9% as determined gravimetrically. Sugar analysis showed that the only sugar residue present in the extract was glucose. Three fractions of the whole extract were obtained by eluting an SPE-column with 50, 60 and 70 % aqueous methanol. Figure 9 shows HPLC chromatograms of the whole extract and the three fractions. The broad peaks were transformed to sharp peaks upon hydrolysis (Figure 8). Thus, the broad nature of the unhydrolysed peaks suggested a variation in *e.g.* molecular weight, substituent and/or substitution pattern. On the basis of peak area, the 60% fraction was the largest fraction (approx. 64%), followed by the 50% fraction (approx. 23%) and the 70% fraction (approx. 13%). The whole extract and its three fractions were similar with regard to the nature of their components absorbing in the UV range used for detection (210-400 nm). However, the relative ratios of the components of the three oligomeric fractions differed. The HPLC chromatogram of the hydrolysed 60% fraction, the major part of the oligomeric extract, was most similar to that of the whole extract. The 70% fraction was rather similar to the 60% fraction but had a slightly higher proportion of SDG compared with other components. The 50% fraction, on the other hand, was clearly different in containing a much smaller proportion of SDG and a much higher proportion of the *p*-coumaric and ferulic acid glucosides. It was possible to observe the same structural variations by ^{1}H NMR analysis. Similar results regarding the relative size and composition of the three fractions were obtained by Strandås *et al.* (2008a).

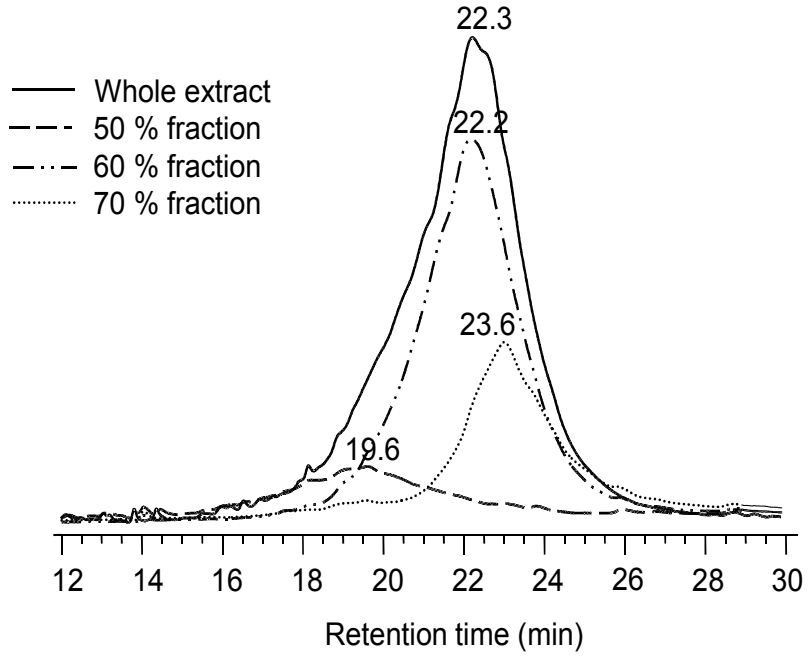

Figure 9. Chromatogram of an unhydrolysed oligomeric flaxseed extract (whole extract) and three fractions obtained by eluting an SPE column with 50, 60 and 70 % aqueous methanol.

Paper III further investigated the structure of the oligomers by performing additional NMR-analyses on the 60% methanolic fraction from SPE of the whole extract. ^{1}H NMR analysis revealed signals corresponding to SDG and probably CoAG and FeAG, as well as the CH_3 and CH_2 groups of 3-hydroxy-3-methylglutaric acid (HMGA; Figure 2). ^{13}C NMR showed peaks for SDG and HMGA residues but no visible peaks for the other phenols (mainly CoAG and FeAG). The carboxylic carbon signal assigned to HMGA was 2 ppm upfield from that of the free HMGA, indicating that HMGA is esterified in symmetrical structures. HSQC-DEPT revealed two types of strong cross peaks, showing that the C-6 of glucose in SDG (Figure 2) was present in both non-esterified and ester-linked forms (Farah *et al.*, 1992). Since some C-6 of the glucose residues in SDG were ester-linked and some were not, some glucose residues must be in terminal position in the oligomer. This finding was confirmed by TOCSY, which showed two types of spin systems for the protons at positions 7, 8 and 9, where signals from the linked SDG were downfield from those of terminal SDG. HMBC

revealed that the ester link is between the carboxylic carbon of HMGA and some of the C-6 of glucose. Taken together, these results suggested a linear and symmetrical structure composed of SDG and HMGA units.

The structure of the oligomer was elucidated from two types of calculations: 1) the ratio of terminal versus linked glucose residues in the oligomer; and 2) the ratio between SDG and HMGA units. The ratio of terminal versus intermediate SDG groups was roughly calculated as 1:4 from the ^{13}C NMR spectrum by integration of the peaks of terminal and linked glucose. Integration of peaks in the ^{1}H NMR spectrum, two protons at positions 8 and four protons at positions 7 of SDG and the $-CH_2-$ groups of HMGA gave an SDG/HMGA ratio of 0.56/0.44. The average numbers of SDG and HMGA molecules in this oligomer were calculated as 4.67 and 3.67, respectively. Both calculations support an average structure of approx. 4000 Da, containing two terminal and eight linked glucose residues (Figure 10). These results are in line with those of Ford *et al.* (2001) which suggest a family of SDG-HMGA dimers, trimers, tetramers and so forth to be present in mature flaxseeds. The work presented in Paper III was the first to reveal the structure of SDG oligomers in flaxseed.

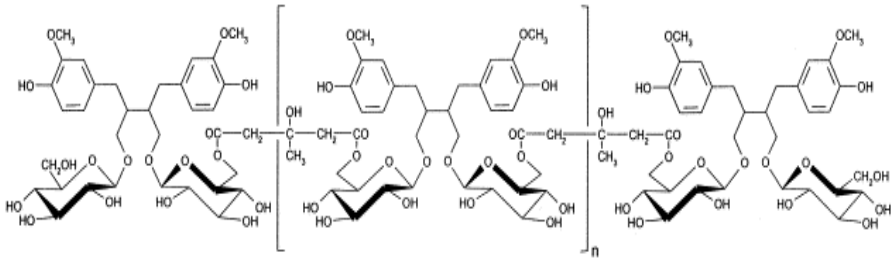

Figure 10. The structure of the flaxseed lignan oligomer (average size, $n = 3$).

4.1.4 Further insights into the nature of the flaxseed lignan oligomer

It was shown recently that the flavonoid herbacetin diglucoside (HDG) is part of the oligomeric structure (Struijs *et al.*, 2007). In addition, as evidenced by NMR analyses, the hydroxycinnamic acids CoAG and FeAG are incorporated into the oligomer directly ester-linked to the C-6 of the glucose units of SDG (Struijs *et al.*, 2008) (Figure 2). Since the linking molecule HMGA also binds to the C-6 position of the glucose moieties (Ford *et al.*, 2001; Struijs *et al.*, 2008; Paper III), the binding of the

hydroxycinnamic acids to this site blocks further growth of the oligomer in that direction. Struijs *et al.* (2009) therefore proposed that the incorporation of CoAG and FeAG determines the size of the oligomer. All previous studies on flaxseed lignan oligomers have been performed using a single cultivar (Ford *et al.*, 2001; Strandås *et al.*, 2008a; Struijs *et al.*, 2009) and thus Paper IV aimed at testing the size determination hypothesis of Struijs *et al.* (2009) by comparing seven different flaxseed cultivars with different SDG to hydroxycinnamic acid ratios.

The molar contributions of SDG, CoAG, FeAG and HDG and the molar ratios of SDG to hydroxycinnamic acids in DFF, as determined by UHPLC analysis of base-hydrolysed extracts, are shown in Table 3. Results from UHPLC analyses of the unhydrolysed oligomers from the different cultivars showed a slight gradual change towards narrower and later eluting peaks as the ratio of SDG to CoAG increased. The results suggest that the oligomeric polarity and molecular size distribution in the different flaxseeds decreases with an increased ratio of SDG to CoAG. GPC of the unhydrolysed oligomer supported the slight gradual change towards higher molecular size with an increasing ratio of SDG and CoAG.

By fractionating the lignan oligomer of flaxseed according to polarity using solid phase extraction with 50, 60 and 70 % aqueous methanol, it was shown that the majority of the oligomeric extract ended up in the 60% methanolic fraction (Table 4), in accordance with previous results (Strandås *et al.*, 2008a; Paper II). Separation of the oligomeric extracts according to polarity and size showed that the 50% fraction contained the smallest molecules and had the highest proportion of hydroxycinnamic acids of the three fractions (Strandås *et al.*, 2008a). Thus, the decreasing yield of the 50% fraction with increasing SDG/CoAG (Table 4) provides further evidence that cultivars with high SDG to hydroxycinnamic acid ratio contain oligomers with lower polarity and higher molecular weight.

MALDI-TOF MS analyses of the seven cultivars did not show a gradual increase in molecular size with increased SDG/CoAG ratio, but they suggested that Vimy, the cultivar having the largest SDG to hydroxycinnamic acid ratio, had a higher average mass range than the other six cultivars, which were similar to each other (Figure 11).

Table 3. *Molar composition of the lignan oligomer from different flaxseed cultivars*

Cultivar	Molar composition, %				Molar ratio	
	CoAG	FeAG	HDG	SDG	SDG/CoAG	SDG/HCA
Early Bird	43	20	4	33	0.8	0.5
Taurus	35	18	4	43	1.2	0.8
Biltstar	23	23	4	50	2.2	1.1
Agraf 17	21	15	5	59	2.8	1.6
Talon	21	12	4	63	3.0	1.9
Niagara	13	24	6	58	4.5	1.6
Vimy	3	8	6	83	27.7	7.5

CoAG = *p*-coumaric acid glucoside, *FeAG* = ferulic acid glucoside, *HDG* = herbacetin diglucoside, *SDG* = secoisolariciresinol diglucoside, *HCA* = hydroxycinnamic acids (*i.e.* CoAG and FeAG)

Table 4. *Percentage yield of the unhydrolysed flaxseed lignan oligomeric extract after fractionation by C18 solid phase extraction (SPE) with 50, 60 and 70 % aqueous methanol (MeOH). See also Table 3 for SDG to hydroxycinnamic acid ratios*

Cultivar	% yield per methanolic fraction		
	50% MeOH	60% MeOH	70% MeOH
Early Bird	25	65	10
Taurus	15	75	10
Biltstar	16	73	11
Agraf 17	12	79	9
Talon	11	70	19
Niagara	11	70	19
Vimy	4	76	20

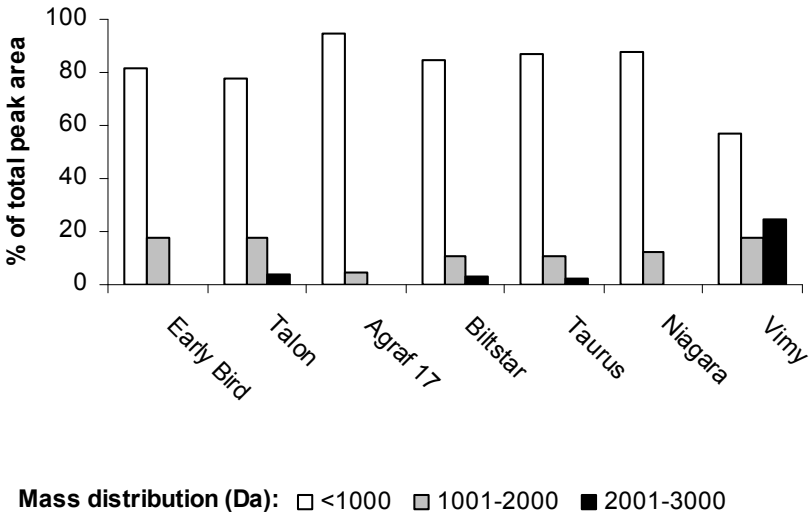

Figure 11. Molecular mass distribution (Da) as a percentage of total peak area from MALDI-TOF MS analyses of the unhydrolysed oligomeric extracts of seven different flaxseed cultivars.

Assuming that all CoAG and FeAG are linked with SDG in oligomeric structures, cv. Vimy should be at least 5458 Da (7.5 SDG + CoAG) (Table 3) which is near the average molecular weight of 4000 Da estimated for another flaxseed sample in Paper III (see above). The range of molecular masses of the oligomers in the other cultivars would then be between 995-1360 Da (min. SDG + CoAG = 994.9 Da to max. SDG + 2 FeAG = 1363.1 Da). This supports in part the MALDI-TOF results obtained for these cultivars in this study, as most peaks in their spectra were below 1500 Da. However, looking at Table 4, where all cultivars have at least 75% of their phenolic compounds in the 60% plus 70% methanolic fractions, and considering the molar composition in Table 2 and the results of Strandås *et al.* (2008a) (see above), it is clear that all hydroxycinnamic acids cannot be bound to C-6 of the glucose units in oligomers containing SDG. It is therefore suggested that there may exist different types of SDG oligomers, e.g. those end-capped by hydroxycinnamic acids at both ends, those end-capped at one end and those not carrying hydroxycinnamic acids at all. In fact, there is NMR evidence that SDG is not always end-capped (Paper III). Some oligomers, of low abundance, may possibly carry glucosylated hydroxycinnamic acids at -OH groups of the glucose units other than C-6.

It is also possible that CoAG and FeAG are involved in other structures than SDG oligomers.

The results suggest that depending on the proportion of monomeric constituents in the seeds, different flaxseeds contain different populations of oligomers, those with high proportions of hydroxycinnamic acids and those with high proportions of SDG. Hydroxycinnamic acids are formed and disappear into bound forms at early stages of seed development, while SDG is formed and bound at later stages (Ford *et al.*, 2001). If the hydroxycinnamic acids terminate the elongation of the oligomer, small oligomers will be formed in early stages of seed development when the content of free hydroxycinnamic acids is high. Larger oligomers with relatively more SDG will be formed in later stages of seed maturation (Struijs *et al.*, 2009

The mass numbers for the two major peaks in cv. Vimy were 709 and 2000 Da. The mass of the major peak in the other six cultivars was 907 Da. These peaks and a few others have not been successfully assigned to any oligomeric structures, suggesting that they originate from compounds that are not part of the oligomeric structure. The MALDI-TOF spectrum obtained from an unhydrolysed extract of flaxseed hulls that was presented by Struijs *et al.* (2009) differed from the spectra obtained from unhydrolysed extracts of DFF in Paper IV in that it seemed to display a higher resolution and higher signal to noise levels. The MALDI-TOF spectra in this study seemed to have been obscured by the presence of interfering compounds to a higher extent, which made it difficult to obtain good signals for the oligomeric constituents.

4.2 Work related to flaxseed oil (Paper V)

4.2.1 Fish white muscle lipid composition

When comparing the TAG fraction of the white muscle of fish fed the FSO+S diet with fish fed the FSO diet, there was a significant decrease in ALA and slight (non-significant) increases in EPA and DHA and the desaturation index (*i.e.* the measure of the conversion ALA to longer and/or more desaturated products) n-3HUFA/ALA. The PFSO and PFSO+S diets gave opposite effects to the FSO and FSO+S diets, respectively. We checked the possibility that treatments were switched during feeding, but this possibility was dismissed since sesamin was only found in muscles of fish that were intended to be fed sesamin. The MO+S diet resulted in slight (non-significant) increases in EPA and DHA compared with the MO diet

(Table 3 in Paper V). In the PL fraction, there were no clear effects of the FSO/FSO+S and PFSO/PFSO+S diets on ALA and DHA, but again, PFSO and PFSO+S consistently behaved in the opposite way to FSO and FSO+S, respectively. The MO+S diet resulted in a significant increase in DHA and DHA/ALA compared with the MO diet (Table 3 in Paper V).

The effects of the FSO+S diet on the fatty acid composition of the white muscle TAG fraction that were observed in Paper V are supported by similar observations in the study of Trattner *et al.* (2008). The effects for TAGs in the MO/MO+S treatments were similar in the two studies but the overall effects were more pronounced in Trattner *et al.* (2008). Possible explanations for the difference are: 1) only four fish per treatment were analysed in Paper V; 2) there was a different fatty acid composition of the flaxseed oils used in the two studies, as the ALA/LA ratio was only 1.8 and 0.6 in the FSO/FSO+S and MO/MO+S diets, respectively, compared with 3.6 and 1.0 in the study by Trattner *et al.* (2008); 3) only sesamin was used in Paper V, whereas an equimixture of sesamin and episesamin was used by Trattner *et al.* (2008). Episesamin has been reported to be a stronger lipid modulator than sesamin in rats (Kushiro *et al.*, 2002).

The flaxseed oil was purified to obtain flaxseed triacylglycerols in order to test the theory that a high ALA/LA ratio and/or minor components in flaxseed oil have an inhibitory effect on desaturation and elongation of ALA. If minor components in flaxseed oil had interfered with elongation and desaturation of ALA, one would have expected the PFSO diet to be more efficient in increasing n-3 desaturation indices than the FSO diet. One could possibly also have expected the PFSO to be more efficient than MO due to higher substrate availability. Moreover, the PFSO+S diet works in the opposite direction to FSO+S. Thus, instead of inhibitory substances in the flaxseed oil, the results from this study point toward synergistic effects between sesamin and minor compounds in the sunflower oil and possibly also in the whole flaxseed oil. The results support the theory that the high ALA/LA ratio in flaxseed oil has a negative effect on n-3 HUFA formation (Tocher *et al.*, 2002). The behaviour of the PFSO+S diet could then be explained by a lack of minor compounds that work synergistically with sesamin, combined with a high level of ALA. However, the stronger effect on the increase in n-3 HUFA observed by Trattner *et al.* (2008) when using flaxseed oil with a higher ALA/LA ratio compared with this study does not support the hypothesis of Tocher *et al.* (2002) that high ALA/LA ratios inhibit the conversion of ALA into n-3 HUFA.

4.2.2 Sesamin and tocopherols

When comparing individual data for rainbow trout white muscle TAGs, a negative correlation (P=0.05) was found between sesamin content and n-3 HUFA/ALA, indicating that increased desaturation and elongation of fatty acids is correlated to decreased muscle sesamin. According to Trattner *et al.* (2008), the MO+S diet resulted in lower tissue sesamin levels than the FSO+S diet. Together, these results suggest that increased n-3 HUFA/ALA is coupled to sesamin metabolism.

Diets containing sesamin were associated with lower levels of γ-tocopherol in white muscle of rainbow trout in Paper V, contradicting studies on rats and humans where sesamin was associated with increased blood and tissue concentrations of γ-tocopherol (Yamashita *et al.*, 1992; Frank *et al.*, 2008). There was also a negative correlation between n-3 HUFA/ALA and γ-tocopherol (P=0.03) in individual muscle PL data, and a tendency for the same in TAGs (P=0.06) in this study. This is in line with a study by Bell *et al.* (2000), where hepatocytes from salmon deficient in α-tocopherol and/or astaxanthin showed an increased ability to elongate and desaturate ALA and EPA.

5 Main findings and conclusions

Work related to the lignan oligomer of flaxseed

- An HPLC method for determination of SDG in flaxseed was developed and used to compare the SDG content of 29 flaxseed cultivars.

- Full spectroscopic data were obtained for 4-O-β-D-glucopyranosyl-*p*-coumaric acid and 4-O-β-D-glucopyranosyl-ferulic acid isolated from flaxseed.

- The linear structure of flaxseed SDG-HMGA oligomers with an average molecular weight of 4 kDa was described for the first time.

- The first solid evidence that flaxseeds of different cultivars contain oligomers of different sizes was presented.

- Results from chromatographic analyses, which were selective for phenolic compounds (recorded at 280 nm), provided support that the molecular size of flaxseed lignan oligomers increases with increasing SDG to CoAG ratios. MALDI-TOF MS results showed that Vimy, a flaxseed cultivar with very low proportions of hydroxycinnamic acids, had a larger average oligomer size than cultivars with higher proportions of hydroxycinnamic acids.

- The size of flaxseed lignan oligomers was shown to be partly influenced by end-capping by hydroxycinnamic acids, possibly at early stages of seed development. It was postulated that biosynthesis of the oligomeric constituents at different stages of seed maturation leads to formation of different populations of oligomers.

Work related to flaxseed oil

- The results obtained in this study confirmed findings by Trattner *et al.* (2008) that sesamin added to diets containing flaxseed oil, or a mixture of flaxseed and sunflower oil, is able to increase the conversion of alpha-linolenic acid to highly unsaturated omega-3 fatty acids (n-3 HUFA) in white muscle of rainbow trout (*Oncorhynchus mykiss*).

- Diets containing purified triacylglycerols from flaxseed oil used for the first time, with and without added sesamin, behaved in the opposite way to whole flaxseed oil with and without sesamin, respectively, in terms of fatty acid composition of white muscle in rainbow trout. The reason for this was unclear, but it may be due to minor bioactive components in vegetable oils working synergistically with sesamin.

- The difference in the ability of sesamin to increase omega-3 highly unsaturated fatty acids (HUFA) in white muscle of rainbow trout when comparing flaxseed oil and a mixture of flaxseed oil and sunflower oil could not be attributed solely to the proposed inhibitory effects of the high ALA/LA ratio of flaxseed oil.

- Both sesamin and γ-tocopherol levels were shown to be inversely related to increased omega-3 HUFA levels in white muscle of rainbow trout.

6 Future perspectives

Work related to the lignan oligomer of flaxseed

- The studies on differences in oligomer size in different flaxseed cultivars need to be followed up by extensive studies comparing the flaxseed cultivar Vimy to other cultivars using hulls instead of whole meal and monitoring biosynthesis during seed development.

- Knowledge about the structure of flaxseed oligomers and their components is of great importance from a nutritional and biochemical point of view and is crucial for complete understanding and possible exploitation.

Work related to flaxseed oil in fish feed

- To evaluate and understand the effects obtained in this work, larger studies with a broader approach, *e.g.* using OMIC technologies, have to be performed.

- In order to understand and compare different studies on the effects of vegetable oil diets on the fatty acid composition of fish, it is necessary to describe the nature of the oils used, *e.g.* level of refinement and minor constituents.

7 References

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