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Composition and properties of flaxseed phenolic oligomers

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Abstract

An extract from flaxseed containing oligomeric structures of the phenolic glucosides secoisolariciresinol diglucoside (SDG), *p*-coumaric acid glucoside and ferulic acid glucoside was fractionated into three oligomeric fractions (F50, F60 and F70) by reversed phase liquid chromatography and further subfractionated by Sepharose CL-6B. The F50 fraction, which had the highest proportion of hydroxycinnamic acid glucosides, was also fractionated on Sephadex LH-20 according to hydrophobicity and size. The different separations resulted in complex profiles of UV-absorbing molecules. HPLC analyses indicated that reversed-phase chromatography separated the oligomers according to composition of the phenolic glucosides, while the subfractionation revealed that other structural features of the oligomers were also important. Using the DPPH radical, SDG and oligomeric fractions showed similar hydrogen-donating abilities comparable to ferulic acid but higher than α-tocopherol, which suggests that SDG was the only active antioxidant.

Keywords: Flaxseed; Phenolic glucosides; Lignan; Secoisolariciresinol diglucoside; Phytoestrogen; Antioxidants

1. Introduction

Phenolic compounds are believed to have protective effects against degenerative diseases in humans, such as cardiovascular diseases, cancer and diabetes mellitus. Flaxseed is a rich source of the lignan secoisolariciresinol diglucoside (SDG, Fig. 2) (Bakke & Klosterman, 1956; Eliasson, Kamal-Eldin, Andersson, & Åman, 2003), which is ester-linked to 3-hydroxy-3-methyl glutaric acid (HMGA) in an oligomeric structure with an average molecular weight of ~4000 Da (Kamal-Eldin et al., 2001). A wide molecular weight distribution of the flaxseed oligomers was reported in the patent of Shukla, Hilaly, and Moore (2004). Other phenolic compounds have also been found in flaxseed, e.g. the hydroxycinnamic acid derivatives, *p*-coumaric acid-4-*O*-glucoside and ferulic acid-4-*O*-glucoside (Johnsson et al., 2002), and the flavonoid herbacetin diglucoside (Struijs et al., 2007). Structures of the oligomers of SDG that are ester-linked by hydroxymethylglutaric acid have been clarified (Ford, Huang, Wang, Davin, & Lewis, 2001; Kamal-Eldin et al., 2001) but structures of oligomers including hydroxycinnamic acid glucosides are still not revealed.

SDG has been reported to have antioxidative effect *in vitro* (Hu, Yuan, & Kitts, 2007) and *in vivo* (Yuan, Rickard, & Thompson, 1999) but we need to study how this antioxidant potential is affected by the oligomeric structure.

In this study, the composition and properties of the dioxane/ethanol-extracted flaxseed phenolic oligomers obtained by fractionation using reversed phase and gel chromatography were investigated, with the focus on the phenolic glucosides SDG, *p*-coumaric acid glucoside and ferulic acid glucoside. In addition, the hydrogen-donating abilities of flaxseed oligomers, SDG and ferulic acid were assayed using the DPPH$^{\cdot}$ inhibition method.

2. Materials and methods

2.1. Chemicals and reagents

Pressed flaxseed cake was obtained from Alternativ Förädling AB (Glanshammar, Sweden). Ferulic acid,

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p-coumaric acid, *o*-coumaric acid, and 1,1-diphenyl-2-picrylhydrazyl (DPPH·) were obtained from Sigma (Sigma-Aldrich Chemie, Taufkirchen, Germany), and α-tocopherol and silica gel 60 (0.040–0.063 mm) were from Merck (Darmstadt, Germany). C18 Bondesil silica (40 μm) was obtained from Varian Inc (Palo Alto, CA, USA), and Sepharose CL-6B and Sephadex LH-20 were from GE Healthcare Biosciences AB (Uppsala, Sweden).

2.2. Isolation of SDG

Flaxseed cake was disintegrated in a kitchen mixer and defatted twice at room temperature with *n*-hexane (1:3, w/v). After drying, the powder was milled (0.5 mm sieve, Retsch type ZM 1, Haan, Germany) to obtain defatted flaxseed flour (DFF).

SDG was extracted from DFF (50 g) by mixing continuously with 60% EtOH (190 ml), water (60 ml) and 2 M NaOH (250 ml) for 16 h at room temperature. The hydrolysate was acidified to pH 3 with 2 M H_2SO_4 (140 ml), and 95% EtOH (1.1 l) was mixed into the solution. After centrifugation (900*g*, 20 min), the liquid phase was evaporated to dryness *in vacuo* at 40 °C and dissolved in 10% MeOH (10 ml 60% MeOH followed by 50 ml water) using an ultrasound bath for 15 min. The extract was loaded onto a Büchner funnel packed with 50 g C18 Bondesil silica (40 μm, i.d. 7 cm, 5 cm high). The material was washed with water (500 ml) and 30% MeOH (200 ml) and an SDG fraction was finally collected with 40% MeOH (400 ml). This eluent was evaporated to dryness *in vacuo* at 40 °C, and redissolved in $CHCl_3$/MeOH/H_2O (100 ml; 6:3:1, v/v/v). The extract was loaded onto a column packed with activated silica gel 60 (i.d. 2.8 cm, 33.5 cm high, column volume 210 ml) and eluted with $CHCl_3$/MeOH/H_2O (10:5:1, v/v/v). The first 100 ml were discarded and thereafter, 20 tubes of 50 ml of eluent were collected. The content of (+) SDG in the fractions was analysed with HPLC and fractions (7–12) were pooled to yield 0.3 g of (+) SDG. HPLC analysis revealed that SDG was the only UV-detectable substance in the isolated material.

2.3. Chromatographic fractionation of the oligomeric extract

2.3.1. Extraction of oligomers

The flaxseed oligomers used in this work were extracted from DFF (150 g) using 1,4-dioxane/95% EtOH (1:1, v/v) (600 ml) in a 2 l container, at 60 °C by continuous stirring overnight. The extract was centrifuged (900*g*, 10 min) twice in 35 ml tubes with intermittent washing with 10 ml of 1,4-dioxane/95% EtOH (1:1, v/v) per tube. The supernatants were combined and evaporated to dryness *in vacuo* at 40 °C.

2.3.2. Solid-phase fractionation of the extracted oligomers on C18

The oligomeric extract was dissolved in 60% MeOH (100 ml), thereafter diluted with water to 30% MeOH (200 ml), and divided into eight portions. Each portion of the extract was fractionated on a Büchner funnel packed with 50 g of C18 Bondesil silica (40 μm, i.d. 7 cm, 5 cm high), which was washed with water (300 ml) and then eluted with 50%, 60% and 70% MeOH (300 ml each) to yield the F50, F60 and F70 fractions, respectively (Fig. 1). Before loading a new portion of extract, the material was washed with MeOH (300 ml) and reconditioned with water. The F50, F60 and F70 fractions were pooled separately, evaporated to dryness and dissolved in 80% EtOH (50 ml each). The fractions were analysed by HPLC as whole oligomers and after alkaline hydrolysis as the phenolic glucosides (+) SDG, *p*-coumaric acid glucoside and ferulic acid glucoside. ^{1}H NMR spectra of F50, F60 and F70 were analysed as described by Johnsson et al. (2002).

2.3.3. Fractionation of F50, F60 and F70 on Sepharose CL-6B

Portions of F50, F60 and F70 (containing 1 mg of total phenolic glucosides each) were dissolved in 1 ml of 80% EtOH) and fractionated at room temperature on a column (i. d. 1.6 cm, 60 cm high) packed with Sepharose CL-6B using 80% EtOH at a flow rate of 0.4 ml min^{-1} as eluent. The fractionation was followed by a UV detector at 280 nm and fractions of 4 ml were collected. F60 and F70 were divided into six fractions and F50 into four fractions and the fractions were analysed for the phenolic glucosides after alkaline hydrolysis by reversed phase HPLC as described below. The total volume of the column was obtained by injection of water. All samples were run in duplicate.

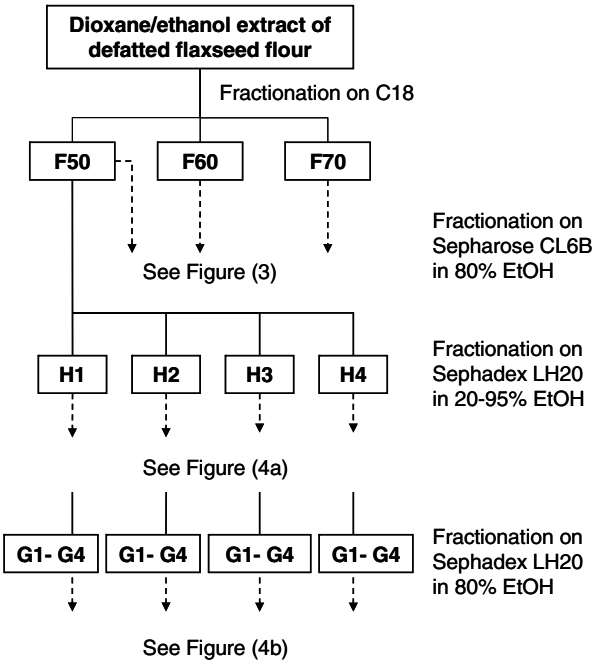


Fig. 1. Scheme of fractionation.

2.3.4. Fractionation of F50 on Sephadex LH-20

Five portions of F50 (containing 0.6 mg of the phenolic glucosides dissolved in 1 ml of 20% EtOH) were each fractionated at room temperature on a column packed with Sephadex LH-20 (i.d. 1.6 cm, 25 cm high) according to hydrophobicity using isocratic 20% EtOH (0–101 ml), followed by 95% EtOH (101–184 ml) at a flow rate of 0.5 ml min^{-1}. The fractionation was followed by UV detection at 280 nm and fractions of 2 ml were collected. The F50 was divided into four fractions (H1: 10–64 ml, H2: 64–104 ml, H3: 104–144 ml, H4: 144–184 ml), evaporated to dryness *in vacuo* at 40 °C and dissolved in 1 ml of 80% EtOH. The fractions (H1, H2, H3, H4) were further fractionated on Sephadex LH-20 according to size by gel filtration using isocratic 80% EtOH. Each H-fraction was divided into four subfractions (G1:10–20 ml, G2: 20–30 ml, G3: 30–40 ml, G4: 40–70 ml). The contents and relative ratios of SDG, *p*-coumaric acid glucoside and ferulic acid glucoside in all 16 fractions were obtained after alkaline hydrolysis by reversed phase HPLC as described below. The total volume of the column was obtained by injection of ferulic acid (0.2 mg in 4.5 ml of 80% EtOH).

2.4. High-performance liquid chromatography analysis of phenolic glucosides

The sample was mixed with 100 μl of internal standard (*o*-coumaric acid, 7.7 μg in methanol) and evaporated to dryness *in vacuo* at 40 °C. The evaporated sample was mixed with 300 μl of distilled water and 300 μl of aqueous sodium hydroxide (2 M) for 1 h at 20 °C. The hydrolysate was acidified to pH 3 using 168 μl of sulphuric acid (2 M), mixed with 9 ml of MeOH and left at room temperature for at least 10 min. After centrifugation (2800*g*, 10 min) in 10 ml tubes, the supernatant was evaporated to dryness *in vacuo* at 40 °C and dissolved in 200–300 μl of MeOH. Before HPLC analysis, the sample was filtered through a GHP Acrodisc® 13 mm syringe filter with 0.45 μm GHP membrane.

A Dionex PDA-100 (Dionex, Sunnyvale, CD, USA) HPLC apparatus with a UV–vis diode array detector and Chromelion software was used for the HPLC analysis as described previously (Eliasson et al., 2003). The injection volume was 10 μl for the oligomeric fractions and 30–60 μl for the phenolic glucosides. The separation of F50, F60 and F70 was performed using a gradient of mobile phases A:B (v/v): 0 min (100:0), 10 min (80:20), 30 min (50:50) and 33 min (30:70) at a flow rate of 1 ml min^{-1}.

2.5. Hydrogen donation ability

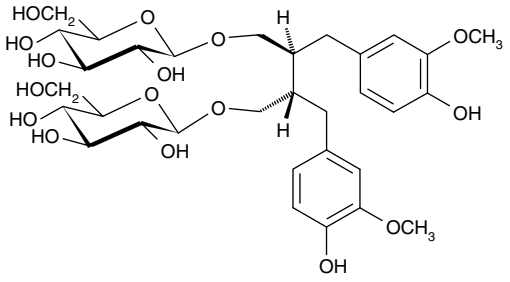
The ability of F50, F60, F70, SDG, ferulic acid and α-tocopherol to transfer hydrogen atoms to 1,1-diphenyl-2-picrylhydrazyl (DPPH·) was studied using a modified method of Brand-Williams, Cuvelier, and Berset (1995). Freshly prepared DPPH· (3 ml, 60 μM in 80% MeOH) was mixed with 25–250 μl of sample in the cuvette. Due to solubility problems of the oligomeric fractions in 100% MeOH, all samples except ferulic acid and α-tocopherol were reacted with DPPH· in 80% MeOH. The ability of the antioxidants to transfer phenolic hydrogen atoms to DPPH· was expressed as the percentage of inhibition of DPPH· after 30 min against the mole amount of SDG in the oligomeric fractions and SDG. All samples were run in triplicate.

3. Results

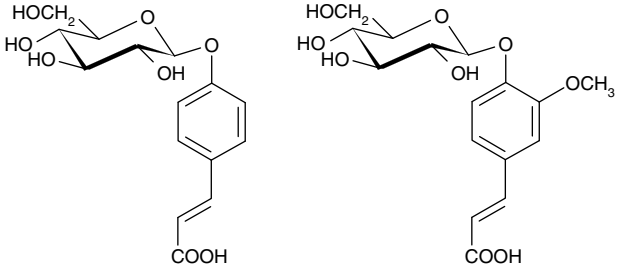
3.1. Content of phenolic glucosides in flaxseed extracts

Defatted flaxseed flour (DFF) was analysed by HPLC after direct alkaline hydrolysis and was found to contain the phenolic glucosides, SDG, *p*-coumaric acid glucoside, and ferulic acid glucoside (Fig. 2) at levels of 2.25, 0.54, and 0.28 g × 100 g^{-1} DFF, respectively. The flaxseed phenolic oligomers containing the phenolic glucosides were extracted from DFF with dioxane/ethanol and fractionated by C18 solid phase extraction into three fractions (F50, F60, and F70) that were analysed by HPLC-DAD before and after alkaline hydrolysis. Unhydrolysed oligomers in F50, F60 and F70 were eluted with peak maxima at 19.4, 22.1 and 23.0 min, respectively, as reported previously (Frank et al., 2004) and with UV–vis absorption maxima at 290, 284 and 283 nm, respectively.

Despite a lower yield, the relative composition of the phenolic glucosides in F50, F60 and F70 was similar to that obtained by direct alkaline hydrolysis of DFF (Table 1).



SDG



p-Coumaric acid glucoside Ferulic acid glucoside

Fig. 2. The chemical structures of the main phenolic glucosides in flaxseed: (+) secoisolariciresinol diglucoside (SDG), *p*-coumaric acid glucoside and ferulic acid glucoside.

Table 1
Total content and relative composition (%) of the phenolic glucosides (SDG, *p*-coumaric acid glucoside and ferulic acid glucoside) in each oligomeric fraction (F50, F60 and F70)

Fraction	Total content of phenolic glucosides ($g \times 100\ g^{-1}$ DFF)	Relative composition (%)		
		SDG	*p*-Coumaric acid glucoside	Ferulic acid glucoside
F50	0.09	50	27	22
F60	0.83	77	12	11
F70	0.39	87	6	7
Total[a]	1.31	78	11	11

[a] Calculated from the contents and the relative composition of phenolic glucosides in each oligomeric fraction.

HPLC analysis of the hydrolysed fractions showed that F60 was the major oligomeric fraction and it had a relative composition of phenolic glucosides similar to that of DFF. F50 was a minor fraction but had the highest relative composition of hydroxycinnamic acid derivatives. ^{1}H NMR spectra of F50, F60 and F70 (results not shown) were similar to those reported by Johnsson et al. (2002), and showed that F60 and F70 had similar compositions, while F50 had proportionally less SDG and more hydroxycinnamic acid derivatives.

3.2. Fractionation of F50, F60 and F70 on Sepharose CL-6B

3.2.1. General

The oligomeric fractions F50, F60 and F70 were separated on Sepharose CL-6B, which separates globular proteins in the molecular weight range of 1×10^4–4×10^6 (Fig. 3). A wide range of molecular sizes was indicated for F60 and F70, but a large portion of the UV-absorbing material in these fractions was eluted after the total volume of the column, showing that size-exclusion was not the only type of chromatography occurring in the system. The F50 chromatogram showed a large peak after the total volume of the column and HPLC results revealed the presence of phenolic glucosides. The relative compositions of phenolic glucosides in the collected fractions of F50, F60 and F70 were stable during the gel filtration, except for the first fraction of F50 (1% of the three phenolic glucosides detected in F50), which had a high proportion of SDG and a low proportion of *p*-coumaric acid glucoside and ferulic acid glucoside.

3.2.2. Fractionation of F50 on Sephadex LH-20

The F50 fraction, containing the highest proportion of hydroxycinnamic acid derivatives, was initially eluted with 20% ethanol concentrations on Sephadex LH-20 with the intention of focussing on hydrophobicity. Four fractions were collected: H1 and H2 in 20% ethanol and H3 and H4 after switching to 95% ethanol (Fig. 4). Each of these fractions was further separated, on the basis of size on LH-20 using 80% ethanol concentration, into four subfractions (G1, G2, G3, G4), ranging from the largest (G1) to the smallest (G4). By comparing the relative composition

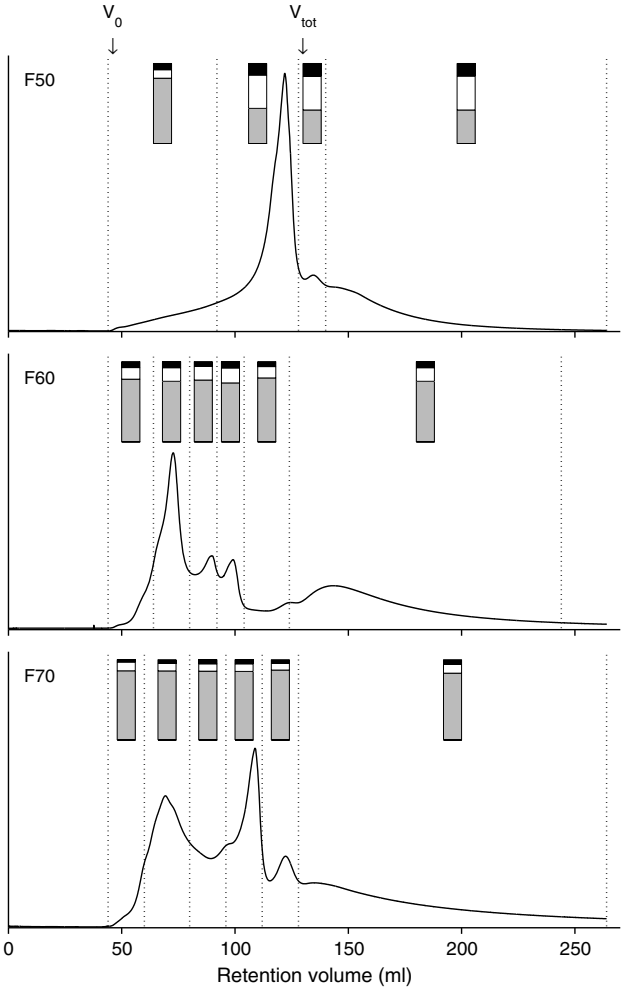


Fig. 3. UV chromatograms at 280 nm of F50, F60 and F70 separated on Sepharose CL-6B using 80% EtOH. The relative composition (%) of the phenolic compounds (SDG ■, *p*-coumaric acid glucoside □, and ferulic acid glucoside ■) of the collected fractions of F50, F60 and F70 analysed by HPLC after base hydrolysis. V_0 is the void volume of the column and V_{tot} is the total elution volume of the column.

of the phenolic glucosides in the F50 subfractions, it was found that small size oligomers (G4) had larger variation in the relative composition than had large size oligomers (G1) (Fig. 5). In the least hydrophobic oligomers (H1), the proportion of SDG increased with decreasing size of oligomer. In the most hydrophobic oligomers (H4), the relative ratio of SDG decreased with decreasing size of oligomer. The fractions H1G1 and H1G2 had high molecular size and least hydrophobic properties. These fractions constituted 49% of F50 and had relative phenolic glucoside compositions similar to F50. Among the most hydrophobic oligomers, H4G4 (1.2% of F50) had a lower molecular size and higher relative content of hydroxycinnamic acid glucosides compared with SDG.

3.3. Hydrogen-donating ability using DPPH·

Reduction of DPPH· in reaction with the oligomeric fractions F50, F60 and F70 was studied over time and com-

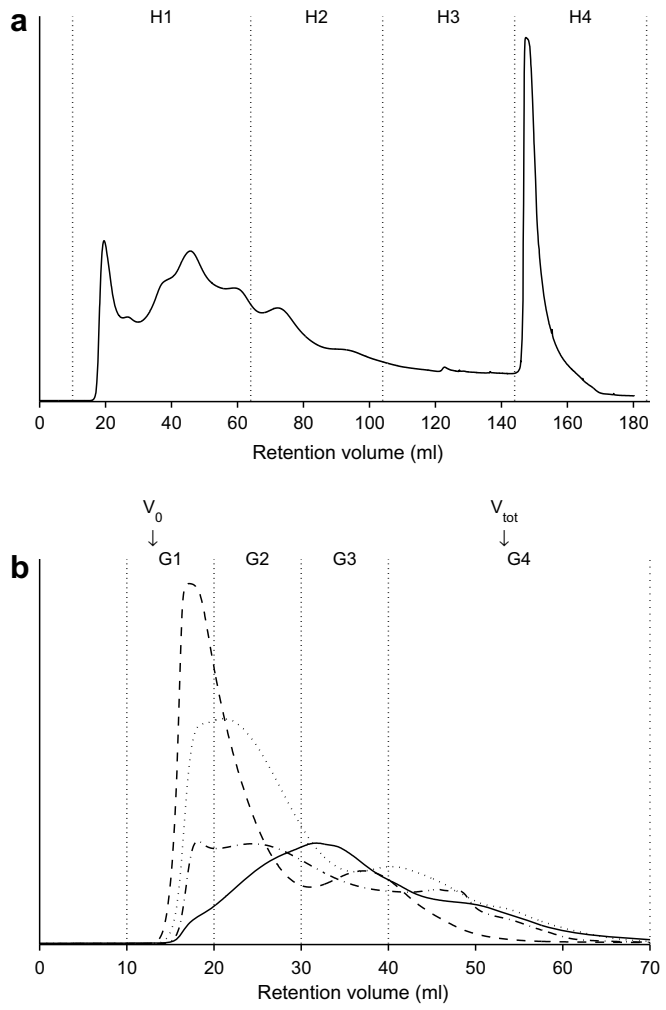


Fig. 4. UV chromatograms at 280 nm of fractionation of F50 on Sephadex LH-20. (a) Fractionation of F50 according to hydrophobicity using isocratic 20% EtOH (H1, H2) and 95% EtOH (H3, H4), and (b) subfractionation of the fractions (H1 – –, H2 ····, H3 – · –, H4 —) using isocratic 80% EtOH (G1, G2, G3, G4). V_0 is the void volume of the column and V_{tot} is the total elution volume of the column.

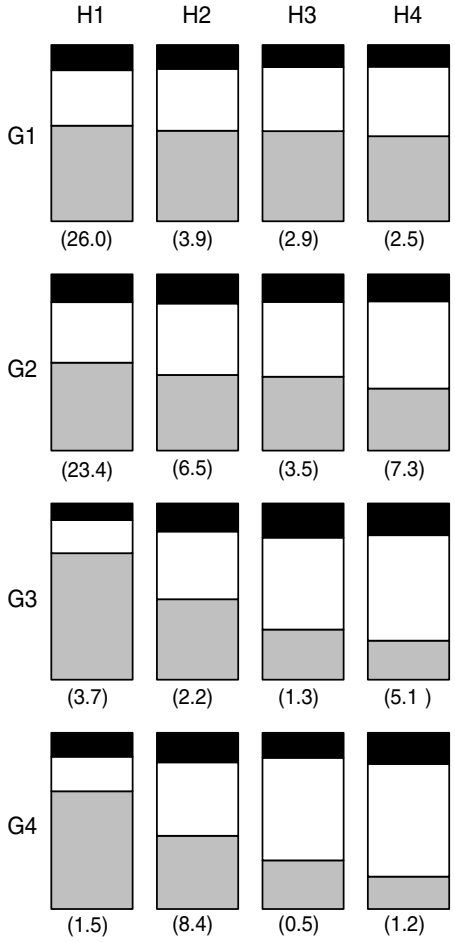


Fig. 5. Fractionation of F50 on Sephadex LH-20 according to hydrophobicity (H1, H2, H3, H4) and gel filtration (G1, G2, G3, G4). The relative proportions of the 16 fractions are shown within brackets and the phenolic compounds in each fraction were SDG ■, *p*-coumaric acid glucoside □, and ferulic acid glucoside ■.

pared with reduction of DPPH· with SDG, ferulic acid and α-tocopherol. The inhibition of DPPH· (%), as a function of the amount (n mole) of SDG in pure form and in the oligomeric fractions after 30 min, is presented in Fig. 6. The inhibitions of DPPH· (%) by pure SDG and oligomers were similar, an increase at low amounts of lignans, followed by declining increases at higher amounts of lignans. Compared with pure SDG, the oligomeric fractions had slightly lower hydrogen-donating ability. F50, which contained small oligomeric fragments, had a stronger hydrogen-donating ability than had F60 and F70 in low concentration range. In the reaction with DPPH·, ferulic acid had comparable and α-tocopherol had lower hydrogen-donating ability than had SDG.

4. Discussion

Knowledge of the structure of the flaxseed phenolic oligomers, especially those containing the 4-*O*-glucosides of *p*-coumaric acid and ferulic acid, is lacking. Using different chromatography techniques, we attempted to further describe the complex structure of these oligomers. 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. In Sepharose CL-6B, the separation was not affected by the relative levels of the phenolic glucosides and thus other structural features of the oligomers were important for the fractionation. UV-absorbing materials eluting after the total volume of the column might be the result of hydrogen bonding between the stationary phase and unknown compounds of hydrophobic nature in the oligomers.

Using Sephadex LH-20, the intention was to separate fraction F50 on the basis of hydrophobicity, using a low concentration of ethanol, and to further separate the subfractions on the basis of size, using a high concentration of ethanol. However, the results indicated that both mobile phases fractionated according to both size and hydrophobicity. Moreover, the proportion of SDG decreased with

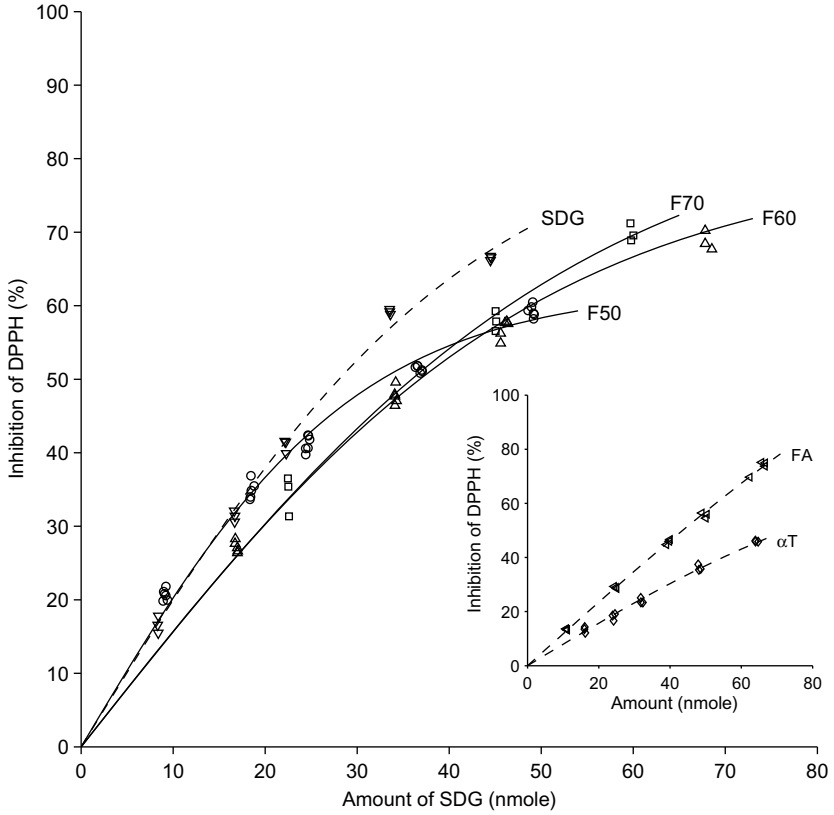


Fig. 6. Inhibition of DPPH· (%) after 30 min of incubation with SDG, F50, F60, and F70 in 80% methanol, and α-tocopherol and ferulic acid in 100% methanol. The total amount of SDG in F50, F60 and F70 was calculated on a molar basis.

increasing hydrophobicity, in contrast to the results from reversed phase chromatography.

The results indicate that F50 contains low molecular size oligomers with less hydrophobic properties and different proportions of the phenolic glucosides than do F60 and F70. In Sepharose CL-6B, the first fraction of F50 might be a contamination from the F60 fraction. The inability to separate SDG and hydroxycinnamic derivatives during the chromatography of F50, F60 and F70 might suggest either a connection or an overlap between the SDG-HMGA oligomers and the hydroxycinnamic derivatives in their oligomeric structures (Fig. 3).

The hydrogen-donating ability of the oligomeric fractions to DPPH· was 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. The lower hydrogen-donating ability of F50, F60 and F70 than SDG might be caused by steric hindrance in the oligomers, i.e. the SDG units within the oligomers not being equally available to DPPH· and/or by interactions with other molecules in the oligomeric preparations. A lower ability of polyphenolic oligomers compared with monomers to donate hydrogens to DPPH· has been reported previously (Goupy, Dufour, Loonis, & Dangles, 2003). The faster reaction rate of F50 than F60 and F70 might be due to more exposed SDG units in low molecular size oligomers or to differences in solubility.

In this study, it was possible to separate the flaxseed oligomers into fractions with distinctively different hydrophobic properties and compositions of the main phenolic glucosides. However, it was not possible to separate the SDG oligomers from the other phenolic glucosides, which indicates a considerable complexity in the structural features of the flaxseed oligomers. Factors such as oligomer size, linkage patterns and interactions with other components may contribute to the observed complexity and the behaviour during chromatographic separations.

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