HPLC Method for Analysis of Secoisolariciresinol Diglucoside in Flaxseeds

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A method was developed for the analysis of secoisolariciresinol diglucoside (SDG) in flaxseeds. The analytical method involves extraction of defatted flaxseed flour with dioxane/ethanol, aqueous base-hydrolysis, solid-phase purification of an SDG-containing fraction, and quantitative analysis by high-performance liquid chromatography (HPLC). Pure SDG was isolated from flaxseed using different column chromatographic methods and used, as external standard, for the calibration of the method and for quantification. The method was then applied to study the variation in SDG content in flaxseeds from 14 cultivars grown in Sweden and 15 cultivars grown in Denmark. The SDG content varied between 11.7 and 24.1 mg/g in defatted flaxseed flour and between 6.1 and 13.3 mg/g in whole flaxseeds.

Keywords: *Secoisolarciresinol diglucoside; lignan; flaxseed; HPLC*

INTRODUCTION

Currently, there is a great focus on the relation between diet and degenerative diseases such as cancer and cardiovascular diseases. Within this line of interest, a relationship was established between phytoestrogens and hormone-dependent cancers (Adlercreutz and Mazur, 1997). Analyses of various foods have shown flaxseed to be the richest source of two types of lignans: secoisolariciresinol and matairesinol (Harris and Haggerty, 1993; Adlercreutz and Mazur, 1997; Mazur and Adlerceutz, 1998). These lignans are believed to exert phytoestrogenic effects through their mammalian metabolites: enterodiol and enterolactone (Boriello et al., 1985; Setchell et al., 1980).

Previous work with flaxseed showed that secoisolariciresinol (SECO) occurs in the form of a secoisolariciresinol diglucoside (SDG, Figure 1) as part of a polymer of unknown composition (Bakke and Klosterman, 1956; Axelson et al., 1982; Thompson et al., 1991; Harris and Haggerty, 1993; Bambagiotti-Alberti et al., 1994a,b; Obermeyer et al., 1995). A few methods for the analysis of SECO or SDG in flaxseeds have been developed (Thompson et al., 1991; Obermeyer et al., 1995; Mazur and Adlercreutz, 1998). Thompson et al. (1991) used fecal culture to convert lignans from various foods into enterolactone or enterodiol which are analyzed by gas chromatography. Obermeyer et al. (1995) developed a high-performance liquid chromatography (HPLC) method for the analysis of SECO released by β-glucuronidase hydrolysis. Mazur and Adlercruetz (1998) used acid hydrolysis to release the glucose residues, and then they analyzed the freed aglycones by isotope-dilution gas chromatography−mass spectrometry. Although the last method gives reliable results, it involves a very lengthy procedure and is highly dependent on the use of isotope-labeled lignans with high cost and limited availability. Muir and Westcott (1996), on the other hand, analyzed SDG by HPLC after base hydrolysis of the polymer.

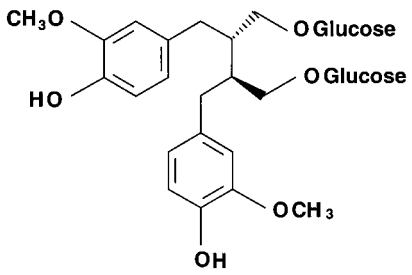

Figure 1. Structure of secoisolariciresinol diglucoside (SDG; 2,3-bis[(4-hydroxy-3-methoxyphenyl)methyl]-1,4-butane-diglucoside).

This paper describes a method, modified from previous methods, for the analysis of SDG in flaxseed. This method involves steps of extraction, base hydrolysis, solid-phase extraction, and HPLC. The method was then used to study the variation in SDG content in flaxseeds from 14 cultivars grown in Sweden and 15 cultivars grown in Denmark.

MATERIALS AND METHODS

Chemicals and Reagents. A reference sample of SDG was obtained as a gift from Dr. Alister Muir (Department of Crop Utilization, Saskatoon Research Center, Canada). All solvents and reagents, used without further purification, were of analytical grade (E. Merck, Darmstadt, Germany).

Flaxseed Samples. A flaxseed cake, from Alternativ Förädling AB (Glanshammar, Sweden), was used for method development. The sample was defatted by several extractions with hexane and dichloromethane, and the defatted flaxseed flour (DFF) was used for method development and for isolation of pure SDG. Swedish and Danish flaxseeds, of known oil content, were provided by Jan Meyer (Oil Crop Department, Svalöv, Weibull AB, Sweden) and Betina Viola Sørensen

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(Danish Institute of Agricultural Sciences). The variation in SDG content was studied using 14 flaxseed cultivars grown in 1997 in Svalöv (Skåne, Sweden) and 15 flaxseed cultivars grown in 1998 in Flakkebjerg (Vest-Sjælland, Denmark). The oil contents in the Swedish flaxseeds were determined after extraction with heptane-2-propanol in steel tubes (Appelqvist, 1968), and the oil contents in the Danish samples were determined after extraction with diethyl ether in a Soxhlet apparatus for 6 h. Both methods were shown to give comparable oil contents in flaxseeds (Appelqvist, 1967).

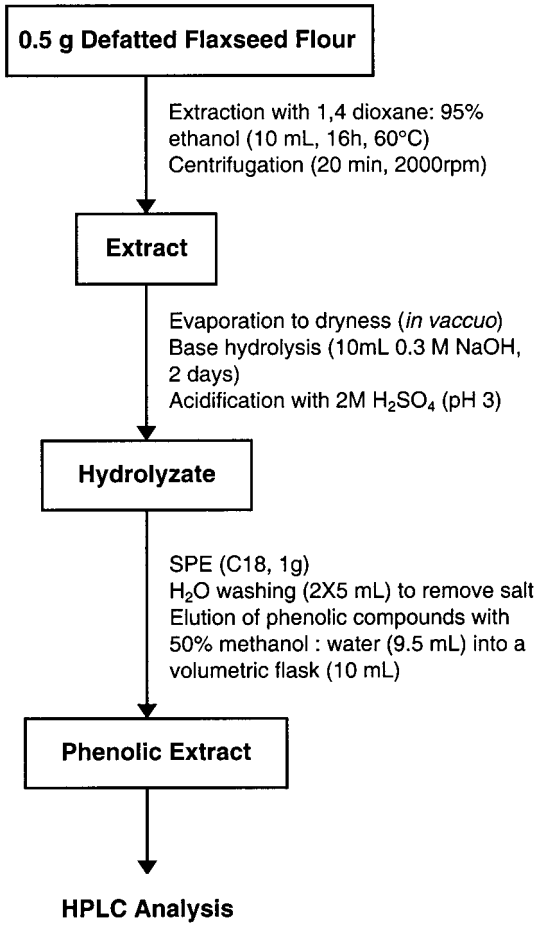
Isolation of Pure SDG from Flaxseed Cake. A SDG-rich fraction was isolated from DFF (100 g) by extraction with 800 mL of 1,4-dioxane/95% ethanol (1:1, v/v) for 2 days at 60 °C (Bakke and Klosterman, 1956). Solvents were evaporated, and the extract was subjected to base hydrolysis (500 mL, 0.3 M aqueous sodium hydroxide) for 2 days at room temperature followed by acidification to pH 3 using 2 M sulfuric acid. The hydrolyzed extract was then passed through a reversed-phase column (3 × 4 cm, Isolute C18, 40−70 μm particle size, 60 nm pore diameter) which was then washed with water (25 mL), and phenolic compounds were eluted with methanol (50 mL). The methanol fraction was concentrated in vaccuo and the residue was applied on a column of silica gel 60 (2 × 20 cm; 32−65 μm particle size, E. Merck) and eluted using chloroform/methanol/water (10:5:1, v/v/v) yielding 117 mg of SDG. The structure of SDG was confirmed by proton nuclear magnetic resonance spectroscopy (^{1}H NMR) performed at 30 °C using CD_3OD as solvent on a 400 MHz instrument (Bruker DRX 400, Karlsruhe, Germany). The spectrum was found to be indistinguishable from that of the reference compound and was comparable to that described by Qiu et al. (1999), and the purity of isolated SDG was >99% on the basis of NMR.

Extraction of SDG from the Defatted Flaxseed Flour. Portions (0.5 g) of the defatted flaxseed flour (DFF) were extracted with 10 mL of 1,4-dioxane/95% ethanol (1:1, v/v) in test tubes by shaking for 16 h in a 60 °C waterbath. After centrifugation (2000 rpm, 20 min), washing, recentrifugation, and in vaccuo evaporation of the liquid phase at 40 °C, the extracts were subjected to alkaline hydrolysis for 2 days under constant rotation using 0.3 M aqueous sodium hydroxide. The hydrolysis conditions are basically similar to those of Rickard et al. (1996). After hydrolysis, the samples were acidified to pH 3 using 2 M sulfuric acid, and their volume was adjusted to 25 mL in volumetric flasks.

Solid-Phase Extraction (SPE) of an SDG-Rich Fraction. Before HPLC analysis, salt was removed from the samples using C18 reversed-SPE columns (Mega Bond Elut, 6 mL/1 g, Varian, Harbor City, CA) conditioned with 5 mL of methanol followed by 5 mL of water. Portions of the hydrolyzed samples (5 mL) were passed through the conditioned columns which were then washed with water (2 × 5 mL) to remove the salt. The SDG-rich fraction was then eluted with 9.5 mL of 50% aqueous methanol and the volume was adjusted to 10 mL in volumetric flasks. The recovery of SDG from the SPE column was >99.5% as judged by quadruplicate determination using standard samples.

High-Performance Liquid Chromatography (HPLC). HPLC analyses were performed on an HP series 1100 system (Hewlett-Packard, Avondale, PA) set to UV/DAD detection between 210 and 400 nm. Chromatograms were recorded at 280 nm, and peaks were integrated using HP Chemstation software. The column was an Econosil RP C18, 5 μm, 250 × 4.6 mm (Alltech, Deerfield, IL). Column thermostat was set to 25 °C and injection volume was set to 10 μL. The mobile phase consisted of 5% acetonitrile in 0.01 M phosphate buffer, pH 2.8 (solvent A) and acetonitrile (solvent B) mixed A/B (v/v): 0 min (100:0), 30 min (70:30), and 32 min (30:70), and was used at a flow rate of 1 mL/min. The SDG peaks were identified and quantified by comparison with those of standard SDG (vide supra). A linear HPLC calibration curve for standard SDG was obtained for the concentration range 0, 20, 40, 80, 120, and 160 μg/mL (R value 0.997). The repeatability of the whole method was tested by simultaneous analysis of 6 replicates, and the coefficient of variation was <5%.

Scheme 1. Analytical Methodology



RESULTS AND DISCUSSION

Scheme 1 summarizes the analytical method developed for the analysis of SDG in flaxseeds. The flaxseed polymer, containing SDG, was extracted as described by Bakke and Klosterman (1956). After base hydrolysis and release of SDG from its polymer with aqueous NaOH, the extracts were acidified to pH 3 to prevent ionization of the carboxylic and phenolic groups. The acidification led to the formation of significant amounts of salt which had to be removed in order to ease sample handling and to protect the chromatographic column from salt precipitation. Thus, a new step of solid-phase extraction was added where the sample was applied to a reversed-phase column and the salt was removed by washing with water. Finally, a fraction containing SDG was eluted with a mixture of methanol/water (1:1, v/v) and analyzed by HPLC. This step eliminates problems occurring during in vaccuo evaporation of salty samples and extends the lifetime of the chromatographic column.

Figure 2 presents a typical HPLC chromatogram of the base-hydrolyzed extract from flaxseed. The gradient used in this study provides a very good separation for SDG and requires less time compared to other HPLC gradients (e.g., Westcott and Muir, 1996). The peak eluting at ca. 19.5 min was ascribed to SDG after injection of the authentic standard. The first two peaks in the chromatogram, eluting between 12 and 15 min, were tentatively assigned to *p*-coumaric acid glucoside and ferulic acid glucoside by comparison with the chromatograms published by Westcott and Muir (1996) and by interpretation of their UV and LC−MS spectra (unpublished results). Work is in progress to identify

Table 1. Variation in Secoisolariciresinol Diglucoside (SDG) Content in Defatted Flaxseed Flour (DFF) and Whole Flaxseeds of Cultivars Grown in Sweden and Denmark[a]

cultivar	oil in seed[b] (% dry matter)	SDG in DFF (mg/g dry matter)	SDG in whole seed (mg/g dry matter)
	cultivars grown in Sweden		
Antares	44.5	13.1	7.3
Barbara	45.1	20.7	11.4
Bor 18	44.9	15.1	8.3
Dufferin	44.9	14.6	8.0
Flanders	47.6	11.7	6.1
Gold Merchant	50.4	21.0	10.4
Helle	44.9	24.1	13.3
Helmi	44.4	17.0	9.5
Mikael	47.2	22.7	12.0
NorLin	45.5	16.6	9.0
NorMan	47.0	16.9	9.0
Pronto	44.7	14.4	8.0
Vimy	47.4	19.5	10.2
Zoltan	47.4	15.9	8.4
	cultivars grown in Denmark		
Dansk soya	38.5	17.6	10.8
Dänish 21	38.7	17.3	10.6
FDB 126	41.6	17.8	10.4
Göteborg I	37.9	17.1	10.6
Harreskovgård	34.8	16.2	10.6
H. C. Møller La Plata	38.1	18.6	11.5
Herkules	39.0	16.1	9.8
Hør 015	38.2	16.4	10.1
Hør 48	40.6	20.2	12.0
La Plata 1939	42.4	14.5	8.4
Lyngby S. H.	36.9	20.1	12.7
Lyngby 21	40.1	18.2	10.9
Øtofte 199/77	43.5	16.0	9.0
Øtofte 235/47	42.1	14.4	8.4
Øtofte 356	39.6	20.8	12.6

[a] Samples were analyzed in triplicate. The coefficient of analytical variation was <5%. [b] Oil content in the Swedish cultivars was determined after extraction with heptane/2-propanol (2:1, v/v) and in the Danish cultivars after extraction with diethyl ether.

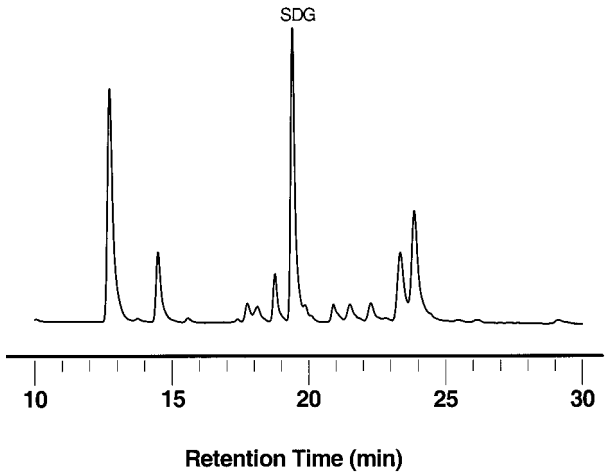


Figure 2. Typical HPLC chromatogram of the hydrolyzate of the flaxseed polymer recorded at 280 nm using dioide array detector (for the structure of SDG, see Figure 1).

the compounds responsible for the other peaks in the chromatogram. Other compounds reported to be present in flaxseed include an SDG isomer (Bambagiotti-Alberti et al., 1994b), pinoresinol diglucoside (Qiu et al, 1999), isolariciresinol (Maegher et al., 1998), matairesinol (Mazur and Adlercreutz, 1998; Maegher et al., 1998), and derivatives of other phenolic acids, e.g., *o*-coumaric, ferulic, *p*-hydroxybenzoic, gentisic, vanillic, and sinapic acids in free and/or bound forms (Kozlowska et al., 1983; Babrowski and Sosulski, 1984). When the SDG polymer was base-hydrolyzed in methanolic rather than aqueous medium, as described by Rickard et al. (1996), the first two peaks in the chromatogram (*p*-coumaric and ferulic acid glucosides) were diminished and some extra peaks eluted after the SDG peak (results not shown). The conversion of these compounds to less polar derivatives in methanol suggests methylation/transmethylation of their carboxylic groups. The yield of SDG was the same whether water or methanol was used as the solvent for hydrolysis, but the separation of HPLC peaks around the SDG peak was much better when hydrolysis was performed in water.

The method developed above was used to study the variation in SDG content in 14 Swedish and 15 Danish flaxseed cultivars grown at one location each. Results (Table 1) show that the level of SDG in these 29 samples varied between 11.7 and 24.1 mg/g in DFF and between 6.1 and 13.3 mg/g in whole flaxseeds. This study is the first to provide data on the variation in SDG content in whole flaxseeds. Without providing SDG levels in all cultivars, Westcott and Muir (1996) reported a range of 13.6-20.0 mg/g for the mean concentrations of SDG in defatted meals from seven Canadian high-linolenic acid cultivars. These authors also found a double-fold variation in flaxseed lignan concentration and noted that the variation was mainly due to cultivation year, with secondary importance to variety, and less importance to cultivation location. The ranges obtained in this study (Table 1) and those obtained by Westcott and Muir (1996) are several times higher than those obtained by Thompson et al. (1997) using microbial fermentation (0.7−2 mg/g in whole seeds). In agreement with the previous findings (Westcott and Muir, 1996; Thompson et al., 1991; Obermeyer et al., 1995; Mazur and Adlercreutz, 1998), results presented in this paper show that flaxseed is an extremely rich source of dietary

lignans and that this seed may have a great potential as an ingredient in possible functional foods. Despite the many reports (Setchell et al., 1980; Borriello et al., 1985; Adlercreutz and Mazur, 1997; Mazur and Adlercreutz, 1998) on the physiological effects of SECO, there is no report available on the nature and utility of the SDG polymer in animal or human nutrition. It is our goal for future research to establish the structure of this polymer and to investigate its potential as a functional ingredient in foods and feeds.

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