Quantitation of the Lignan Secoisolariciresinol Diglucoside in Baked Goods Containing Flax Seed or Flax Meal

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Samples of commercially prepared white, whole wheat, flax, and multigrain breads were analyzed by a rapid RP-HPLC method for the presence of the lignan secoisolariciresinol diglucoside (SDG). SDG was detected only in products containing flax, with concentrations ranging from 0.06 to 1.98 μM/g of DW (19−602 μM/loaf). Full-fat flax meal, powdered aqueous alcohol extracts of flax seed, and SDG were added to a white bread mix and baked into loaves in a domestic bread maker. Quantitative recovery of SDG from the test breads was observed when SDG was added; however, when flax meal or aqueous alcohol extracts were added, only 73−75% of the theoretical yield of SDG was recovered. SDG was also detected in commercially prepared flax cookies, bagels, and muffins with concentrations ranging from 0.26 to 2.93 μM/g of DW. The extent of grinding of the flax seed was also shown to have a significant effect on the recovery of SDG from both flax meal breads and baked goods, with extraction of SDG from finely ground samples greater than that from course material.

Keywords: *Flax seed; lignan; secoisolariciresinol diglucoside; HPLC; bread; SDG*

INTRODUCTION

In recent years there has been increased interest in the effect of flax seed components on human health (Carter, 1993; Clark et al., 1995; Cunnane et al., 1995; Parbtani and Clark, 1995; Serraino and Thompson, 1991, 1992a,b). Although much of this interest has focused on the effects of the omega-3 fatty acids present in high levels in flax seed oil, there has been increasing interest in the nonoil components, particularly the lignan fraction (Thompson et al., 1994; Thompson, 1995). This interest has been driven by the observation that the flax lignan, secoisolariciresinol diglucoside (SDG), appeared to be the principal precursor for the mammalian lignans enterolactone and enterodiol (Axelson et al., 1982). The principal lignan in flax seed, SDG was first isolated from flax seed in 1956 (Bakke and Klosterman, 1956); however, until recent work by our laboratory (Westcott and Muir, 1996), there was no simple or rapid method for the direct quantitative determination of SDG in flax seed or products containing flax seed. A number of other methods of indirect determination of SDG levels have been proposed (Obermeyer et al., 1995; Mazur et al., 1996; Thompson et al., 1997; Nilsson et al., 1997; Nesbitt and Thompson, 1997); however, none of these methods provide a simple direct analysis for the SDG levels in flax seed, flax meal, or products containing flax seed.

At the Saskatoon Research Centre we have been interested in the recovery and purification of SDG from flax seed and flax meal and have succeeded in producing significant quantities of pure SDG (Westcott and Muir, 1998). We have also developed HPLC methods for the quantitation of SDG in flax seed and flax seed meal (Westcott and Muir, 1996). In this paper we describe the adaptation of this HPLC method for the rapid detection and quantitation of SDG in baked goods. Results of preliminary studies were previously reported (Muir and Westcott, 1996).

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MATERIALS AND METHODS.

Solvents, Reagents, and Standards. All solvents used were of HPLC grade. Methanol was obtained from Anachemia Canada (Ville St-Pierre, PQ). Sodium hydroxide and acetic acid were obtained from VWR Canlab (Mississagua, ON). A reference sample of SDG was provided by Dr. W. F. Clark, London Health Science Centre. Secoisolariciresinol was provided by Prof. N. G. Lewis, Washington State University, and later by enzyme hydrolysis of purified SDG. β-Glucuronidase, type H-1 from *Helix pomatia*, was obtained from Sigma-Aldrich Canada (Oakville, ON). The commercial flax meal was provided by United Grain Growers and was generated at the Cargill Mill in Fargo, ND.

Preparation of Samples. Flax bread, white bread, whole wheat bread, flax muffins, flax bagels, and flax cookies were obtained from bakeries in the Saskatoon area between January and July 1996. Whole loaves of bread were divided into three subsamples: a representative sample of the whole loaf, the crust, and the center. Each sample was then freeze-dried and the dry weight of each loaf calculated. The muffins, bagels, and cookies were also freeze-dried. All dried samples were ground in a mortar and pestle. In samples having a significant number of intact flax seeds or large fragments of flax seed, a subsample was ground in a Wiley mill to pass a 20 mesh screen. The powdered samples were then defatted by stirring the sample with 100 mL of hexane/50 g of powder for 1 h in a covered beaker in a fume hood. The hexane was removed by filtration and the defatted powder allowed to air-dry. We were also able to obtain a sample of the flax meal used by one of the commercial bakeries. All meal samples were defatted and, where indicated, ground in a Wiley mill to pass a 20 mesh screen. All samples were analyzed on a dry weight basis.

Isolation of SDG from Flax Meal. The SDG used in these experiments was obtained by extracting flax meal obtained

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from a commercial flax crushing plant. The meal (4 kg) was extracted with 63% v/v aqueous ethanol (30 L) for 4 h at room temperature with stirring. The aqueous alcohol extract was decanted from the meal and the wet meal re-extracted with a further 15 L of aqueous alcohol. The aqueous alcohol extract was separated from the meal by filtration, and the extracts were combined to yield 40 L of extract. A portion of the extract (10 L) was reduced to the aqueous phase and freeze-dried to yield a powdered aqueous alcohol extract. The balance of the extract (30 L) was subjected to base hydrolysis (120 mL of 50% sodium hydroxide, w/w) for 24 h. The hydrolysis reaction was then stopped by adjusting the pH to 6.5 with glacial acetic acid (135 mL) (Westcott and Muir, 1998). The resulting extract was reduced to the aqueous phase by evaporation under reduced pressure and chromatographed on a solid-phase extraction cartridge (15.7 L of C-18 resin), which was washed in turn with 1% acetic acid and 20% ethanol containing 1% acetic acid (v/v). The SDG was eluted from this cartridge with 30% ethanol. The SDG-rich fraction (80−90% SDG) was concentrated to the aqueous phase by evaporation under reduced pressure and freeze-dried. SDG with a purity of >98% was obtained by preparative HPLC on a C-18 radial pack column (Bondapak C-18, 15−20 μm 125 Å, 4 × 31 cm) (Waters Canada, Mississagua, ON) eluted with a 1% aqueous acetic acid (solvent A)/methanol (solvent B) gradient t(min) = 0, A = 90%; t = 2, A = 90%; t = 52, A = 65%; t = 53, A = 65%; t = 65, A = 90%; flow = 50 mL/min and detection at 280 nm). The purity of the sample of SDG incorporated into the bread in this study was determined to be 82% by HPLC analysis.

Analysis of SDG in Flax Meal Samples. The SDG content of full-fat and defatted flax meal was determined as follows. A 0.5 g sample was extracted with methanol/water (70:30, v/v, 10 mL) in a screw-capped test tube in a water bath at 60 °C for 3 h (Westcott and Muir, 1996). The samples were mixed by shaking every 30 min to facilitate extraction because the solids tend to settle out. After 3 h, the supernatant was clarified by centrifugation, and duplicate aliquots (2 mL) were subjected to base hydrolysis (0.5 mL of 0.5 N NaOH). After 3 h at room temperature, the hydrolysis was complete, and the samples were neutralized with acetic acid (0.5 mL of 0.5 N), filtered (0.2 μM), and subjected to HPLC analysis. All samples were analyzed in triplicate.

Analysis of SDG in Bakery Samples. A 4 g defatted sample was extracted with methanol/water (70:30, v/v, 20 mL) in a screw-capped test tube in a water bath at 60 °C for 3 h (Muir and Westcott, 1996). The samples were processed as for the flax meal samples, and all bakery samples were analyzed in triplicate.

HPLC Analysis of SDG. HPLC analysis was conducted on a Waters 2690 Alliance system equipped with a 996 PDA detector operating under Millenium chromatography manager (Waters Canada, Mississagua, ON). SDG was separated from other compounds in the extract by chromatography on a Symmetry C18 Column (5 μm, 250 × 4.6 mm, Waters Canada) eluted with a 1% aqueous acetic acid (solvent A)/methanol (solvent B) gradient (t = 0, A = 95%, B = 5%; t = 44, A = 40%, B = 60%; t = 48, A = 40%, B = 60%; t = 55, A = 95%, B = 5%; detection at 280 nm.) The presence of SDG in the samples was confirmed by standard addition and PDA analysis. The presence of SDG was also confirmed by LC-MS analysis of secoisolariciresinol after hydrolysis with β-glucuronidase (*Helix pomata*; Sigma Aldrich Canada, Oakville, ON), using a Waters Integrity Thermabeam LC-MS (Waters Canada) and negative ion electrospray LC-MS/MS using a Micromass Quattro LC (Micromass Canada, Montreal, PQ).

Standard Flax Bread. To determine the extraction efficiency for SDG from bread products, a series of standard bread loaves were prepared in triplicate using a domestic bread maker (Regal model K6769C). A standard white flour bread mix was used as the base material, and the loaves were prepared according to the instructions provided with the bread maker and baked at a temperature of 135 °C. Each loaf was prepared as follows: 350 g of white flour, 15 mL of vegetable oil, 6.3 g of sugar, 4.5 g of salt, and 3.0 g of yeast. One egg was added to the mix for each loaf unless otherwise indicated. The amount of vegetable oil added was adjusted down in the loaves containing full-fat flax meal to compensate for the extractable oil content of the flax meal. The following samples were prepared: (A) white flour alone; (B) white flour supplemented with 100 mg (145.8 μM) of SDG per loaf; (C) white flour supplemented with powdered aqueous alcohol extract of flax seed [equivalent to 100 mg (145.8 μM) of SDG per loaf]; (D) same as for (C) except no egg was added. In a second series, a sample of commercial vacuum-packed ground whole flax seed was obtained and used to replace an equivalent amount of white flour to create a series of loaves with 4, 8, and 12% full-fat flax meal per loaf. The commercial full-fat flax meal contained a significant number of intact flax seeds and yielded only 24% oil on hexane defatting; however, the oil from intact seeds would not be removed by this defatting procedure. All of the loaves produced were sampled and analyzed as described above.

RESULTS AND DISCUSSION

Lignan Levels in White Bread and Specialty Breads. A total of eight different white and specialty bread examples (three commercial, three domestic, one whole wheat, and one sesame seed) were analyzed for SDG. No SDG was found in any of these samples even after the extract was concentrated 9-fold. No interfering peaks were observed in these chromatograms (Figure 1A), indicating that neither wheat flour nor any other of the ingredients commonly used in bread contained compounds that might interfere with the determination of SDG levels in flax bread.

Lignan Levels in Commercial Breads and Baked Goods Containing Flax. A total of 10 different loaves from seven different bakeries were analyzed for SDG. All product labels indicated that flax seed was included in the bread. When aliquots of the unhydrolyzed extracts prepared from these breads were analyzed by HPLC, no evidence of free SDG was detected (Figure 1B). This is consistent with our previous observations and those of Bambagiotti-Alberti et al. (1994) that SDG does not exist in a free form in flax but rather as an ester-linked component of a polar complex. These results also indicated that the leavening or the baking process did not result in any significant release of free SDG from this polar complex. After alkaline hydrolysis, SDG was found in all 10 samples (Table 1; Figure 1C), however, the range of values varied greatly.

In our previous studies (Westcott and Muir, 1996), we have observed that cultivar and year of harvest have significant effects on the level of SDG present in flax seed and that location has a weak effect. These observations were recently confirmed by Thompson et al. (1997), although the levels reported were significantly lower than we have routinely observed. The concentration of SDG found in some of the flax breads was significantly lower than could be expected if 7−8% flax seed had been used in the preparation of the bread, even if the cultivar with the lowest SDG content had been used.

When a sample of bakery flax meal was analyzed for SDG using the flax seed screening assay (Westcott and Muir, 1996), SDG was found to be present at levels consistent with values observed in the analysis of defatted flax meal. When the values for the SDG content of the flax bread were compared to the estimated SDG content based upon the SDG content of the meal used to bake the bread, the two estimates agreed within 5%.

There was no significant difference between the SDG content of the crust and the core, indicating that SDG can survive the higher temperatures that the crust is exposed to.

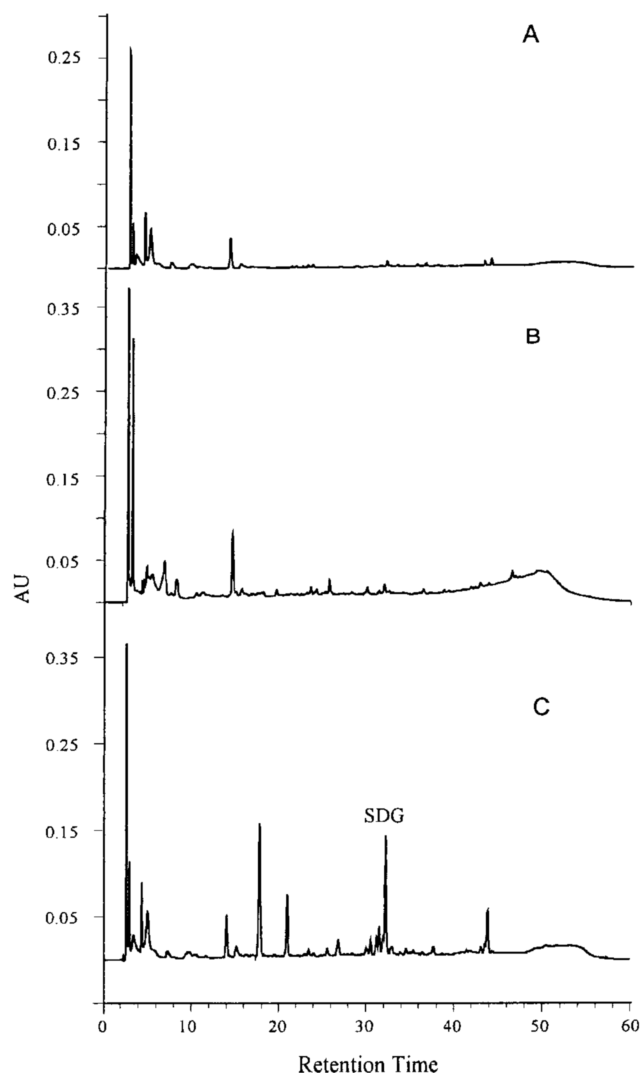

Figure 1. HPLC analysis of aqueous alcohol extracts of bread samples: (A) white bread extract; (B) unhydrolyzed flax bread extract; (C) flax bread extract after base hydrolysis. HPLC conditions were as described in the text. UV detection was at 280 nm.

Table 1. SDG Levels in Commercial Flax Breads Obtained from Retail Shops in Saskatoon

label description	SDG (μM/g of DW)	SDG (μM/loaf)
flax	1.98 ± 0.12[a]	602 ± 32
flax	1.55 ± 0.22	568 ± 80
flax	1.22 ± 0.03	446 ± 13
flax	1.55 ± 0.03	436 ± 6
flax	0.79 ± 0.06	272 ± 22
flax	0.76 ± 0.04	253 ± 15
flax	0.50 ± 0.04	162 ± 15
multigrain	0.69 ± 0.13	200 ± 38
multigrain	0.26 ± 0.04	86 ± 13
multigrain	0.06 ± 0.03	19 ± 13

[a] ± SD, $n = 3$.

Effect of Particle Size on SDG Extraction Efficiency. One possible explanation for the low levels of SDG present in some bread samples was the presence of whole or only partially ground flax seed. To assess the effect of particle size on extraction efficiency, several samples of multigrain bread, in which whole or only partially ground flax seeds were visible, were ground in a mill and the sample was analyzed for SDG as described previously. The results for the ground sample were compared to those of the unground sample, and in all cases (Table 2) the yield of SDG increased with decrease in particle size. However, when flax bread prepared with ground flax seed was reground, there was no significant increase in the extractability of the SDG. Because whole intact flax seeds often pass through the digestive tract intact, the bioavailability of SDG from whole flax seed is likely to be significantly lower than that from ground samples.

Table 2. Effect of Reduction in Particle Size on Extraction of SDG from Flax Bread

label description	SDG (μM/g of DW)	
	before regrinding	after regrinding
multigrain	0.69 ± 0.13[a]	1.01 ± 0.06
multigrain	0.26 ± 0.04	0.57 ± 0.01
multigrain	0.06 ± 0.03	0.15 ± 0.01
flax	0.79 ± 0.06	0.85 ± 0.03

[a] ± SD, $n = 3$.

Recovery of SDG from Flax Bread. To determine the recovery of SDG from flax bread, a series of loaves containing 4, 8, and 12% full-fat flax meal, unhydrolyzed aqueous alcohol extract of defatted flax meal, and 82% pure SDG were prepared. The full-fat flax meal was obtained from a commercial source in the form of vacuum-packed ground flax seed. A sample of the meal was analyzed for SDG according to the method of Westcott and Muir (1996), and the test loaves were analyzed as described previously. When the full-fat ground flax meal was analyzed without additional grinding, the sample was found to contain 7.73 ± 0.54 μM SDG/g or 12.7 ± 0.9 μM/g after defatting. Because this value was considerably lower than that we had previously detected in ground flax seed, a subsample was ground in a Wiley mill and reanalyzed. The reground defatted meal was found to contain 22.59 ± 0.74 μM SDG/g (13.8 ± 0.5 μM/g on a full-fat basis). This highly significant difference can be attributed to the reduction in particle size and consequent increase in extraction efficiency. Recently, Nesbitt and Thompson (1997) reported the total lignan content in raw flax seed to be 1.774 μM/g, which is ∼10% of the SDG level we have routinely detected in flax seed (Westcott and Muir, 1996).

As with the commercial white breads, analysis of test loaves made with white flour did not contain any SDG. In addition, HPLC chromatograms of extracts of white breads before and after base hydrolysis did not indicate the presence of any other compounds that might interfere with the determination of SDG.

When 82% pure SDG was added to test loaves ($n = 3$) made from white flour, a recovery of 99.5 ± 6.5% was achieved, indicating that recovery of SDG was unaffected by the bread-making process. However, as SDG does not occur as a free compound, but rather as a complex ester, recovery of the SDG component of the complex ester was determined both in the presence and in the absence of added egg. Because the SDG ester complex contains a large number of phenolic hydroxyl groups that might be expected to interact with the added protein content of the egg and affect recovery of the SDG, the egg was eliminated from some of the test loaves. SDG recovery was quantitative in both test breads (101.9 ± 5 and 101.6 ± 21.6%; complex ester, with and without egg, respectively), indicating that any interaction between the egg and the SDG complex did not affect SDG recoveries. The absence of the egg in the

Table 3. SDG Levels in Flax Breads Prepared with Known Amounts of a Commercially Available Full-Fat Flax Meal

	actual SDG content[a] (μM)	SDG content of bread by analysis (μM)	% recovery
white flour only	0	0	
white flour + 4% flax meal	154	116 ± 12.3[b]	75.3
white flour + 8% flax meal	313	228 ± 38.6	72.8
white flour + 12% flax meal	463	337 ± 48.6	72.8

[a] The SDG content of the added flax meal was 13.8 ± 0.5 μM/g on a full-fat basis. [b] ± SE, $n = 3$.

Table 4. SDG Levels in Other Baked Goods Containing Flax Seed Obtained from Retail Shops in Saskatoon

description	SDG (μM/g of DW)	SDG (μM/unit)
plain flax cookie	1.66 ± 0.01[a]	39.36 ± 0.04
(reground)	2.24 ± 0.10	53.12 ± 2.48
flax cookie, date filling[b]	2.59 ± 0.15	65.74 ± 3.64
(reground)	2.93 ± 0.03	74.34 ± 0.73
flax cookie	1.20 ± 0.01	62.97 ± 1.02
flax cookie, strawberry filling[b]	1.73 ± 0.01	116.03 ± 0.01
flax bagel	0.85 ± 0.01	68.51 ± 0.01
(reground)	1.18 ± 0.04	96.21 ± 4.23
flax bagel	0.26 ± 0.03	23.18 ± 2.92
flax muffin	1.76 ± 0.07	73.18 ± 2.92

[a] ± SD, $n = 3$. [b] Filling removed prior to analysis.

dough made incorporation of the complex ester more difficult and appeared to account for the greater sampling variability in these loaves.

To determine if the process of bread-making resulted in any liberation of SDG from the ester complex, an aliquot of all extracts was subjected to HPLC analysis before the hydrolysis step. In all cases, no free SDG was found in the unhydrolyzed bread extracts. Samples of dough were also prepared containing the powdered aqueous ethanol flax meal extract and analyzed for SDG. No free SDG was found in the dough samples, and quantitative recovery of the added SDG was achieved after base hydrolysis of the dough extracts (data not shown).

Test loaves prepared with 0, 4, 8, and 12% added flax meal were analyzed for their SDG contents (Table 3). Unlike the addition of SDG to bread, there was a significant and consistent reduction in recovery of SDG from the test loaves when full-fat flax meal was added. Recovery of SDG ranged from 73 to 75% of the theoretical yield of SDG, and the amount of flax meal added did not appear to influence SDG recovery.

SDG in Cookies, Bagels, and Muffins. Flax seed is added to many other baked goods including cookies, bagels, and muffins. In all cases when flax was declared on the label, SDG was found in the sample, although the levels varied widely (Table 4). When the SDG levels were expressed on a dry weight basis, the SDG levels were in the same range as observed for the flax bread samples, indicating that the flax had likely been incorporated into the baking mix in approximately the same proportion as in the bread. As with the flax breads, when whole or partially ground flax seed was present in the product, the yield of SDG increased significantly when the sample was reground.

Conclusion. Both the complex ester form of SDG and free SDG are stable to the bread-making process and are quantitatively recovered from bread. The complex ester form of SDG does not appear to undergo hydrolysis during the preparation and baking of bread. There was a consistent reduction in the recovery of SDG (73−75% of the theoretical yield) from flax breads prepared by the addition of full-fat flax meal. In commercially prepared bread and also in muffins and cookies, there is considerable variation in the amount of SDG present in these products, indicating that the amounts of flax meal being added to these products vary widely. The labels usually did not disclose the amount of flax or flax meal added, only that flax was added. Because the amount of SDG present in flax meal itself is also variable (Westcott and Muir, 1996), it would be desirable in the future to report an actual lignan content in addition to the percentage of flax meal or flax seed incorporated into bread or baked goods to reflect both the variability of the SDG content of the flax seed used and the amount incorporated into the product.

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