Cyanogenic Compounds in Flaxseed

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The seeds of 10 flax cultivars (Andro, Flanders, AC Linora, Linott, McGregor, Noralta, NorLin, NorMan, Somme, and Vimy) grown at Portage la Prairie, MB, in 1987, 1988, and 1989 and at Beaverlodge, AB, and Indian Head, SK, in 1989 were analyzed for content of cyanogenic glucosides by HPLC. The main cyanogenic compound was the diglucoside linustatin at 213–352 mg/100 g of seed, accounting for 54–76% of the total content of cyanogenic glucosides. The content of neolinustatin ranged from 91 to 203 mg/100 g of seed. Linamarin was present at low levels (<32 mg/100 g) in 8 of the 10 cultivars analyzed. The content of all three cyanogenic glucosides was dependent on cultivar, location, and year of production, with cultivar being the most important factor.

INTRODUCTION

Cyanogenic glucosides are nitrogenous secondary plant metabolites derived from amino acids. Their presence in crop species has several important implications. High levels of cyanogenic compounds severely restrict the amount of such meals in animal rations. The possibility of improving the commercial value of some crops by reducing the levels of cyanogenic glucosides has generated considerable interest in understanding their changes in flaxseed.

The major cyanogenic glucosides present in flaxseed have been identified as linustatin and neolinustatin, a β-gentiobioside of acetone cyanohydrin, and methyl ethyl ketone cyanohydrin, respectively (Smith et al., 1980). The presence of a minor component, linamarin (1-cyano-1-methylethyl β-D-glucopyranoside), along with diglucosides linustatin and neolinustatin was detected in linseed meal by thin-layer chromatography (Brimer et al., 1983). Recently, Schilcher and Wilkens-Sauter (1986) found only traces of linamarin in their quantitative determination of cyanogenic glucosides of 48 flax samples using reversed-phase HPLC. These contradictory reports on the presence of linamarin in flaxseed imply limitations in the analytical procedures employed in the extraction, separation, and detection of cyanogenic glucosides.

Bhatty (1990, personal communication) reported strong seasonal effects on the total cyanogenic glucoside of eight flax cultivars grown at several locations in 1987 and 1989. The effect of the environment on flaxseed cyanogenic glucosides was also alluded to by Schilcher and Wilkens-Sauter (1986). However, studies confirming these variabilities on individual cyanogenic glucosides have not been reported.

In this paper, we present a simple, improved reversed-phase HPLC method for the separation and quantitation of cyanogenic glucosides in flax. The distribution of individual cyanogenic glucosides and the genotype and environment effect on their levels were also analyzed in several flax cultivars.

MATERIALS AND METHODS

Seed Samples. Samples of 10 oil-type flax cultivars were obtained from standardized co-operative tests conducted at Portage la Prairie, MB, during 1987, 1988, and 1989 growing seasons according to procedures established by the Western Expert Committee on Grain (Anonymous, 1992). Three of the 10 cultivars, Flanders, Linott, and Noralta, also grown in 1989 in co-operative tests at Indian Head, SK, and Beaverlodge, AB, were used to investigate location effects on cyanogenic glucosides.

Extraction. Whole flaxseed was ground in a coffee grinder and extracted using 70% methanol (Schilcher and Wilkens-Sauter, 1986). For a typical extraction, 1 g of ground full-fat flaxseed was extracted for 30 min at 30 °C in 25 mL of 70% methanol in a sonic water bath. The extract was filtered through a Whatman No. 4 filter paper. A 10-mL aliquot of the extract was dried under vacuum at 30 °C, dissolved in 1 mL of the mobile phase used for the reversed-phase HPLC (95/4.95/0.05 H_2O/MeOH/H_3PO_4), and filtered through a 0.45-μm Millipore filter prior to HPLC analysis.

High-Performance Liquid Chromatography. The flax samples were analyzed according to a modification of the procedure described by Schilcher and Wilkens-Sauter (1986). Samples (20 μL) were analyzed by an HPLC system consisting of an LKB pump Model 2248 and a Model PMV-7 motor valve (Pharmacia, Bromma, Sweden) equipped with a 20-μL loop and a Shimadzu refractive index detector RID-6A (range 32×10^{-6} RIU) interfaced through a PE Nelson 900 series with an IBM personal computer.

An Ultro Pac prepacked column (4 × 250 mm), Lichrosorb RP-18, 10-μm particle size (LKB, Bromma, Sweden), was used with 4.95% MeOH, 0.05% phosphoric acid, and water as mobile phase. The system was operated isocratically at a flow rate of 0.7 mL/min. Separations were performed at 21 ± 1 °C. Typically, a 5-min equilibration period was used between samples, requiring about 35 min/sample.

Identification and Quantitation. The glucosides were identified by comparing their retention times with those of authentic standards. Under these conditions, the retention times of linamarin, linustatin, and neolinustatin were 8.2, 11.0, and 23.6 min, respectively. Quantitation was based on an external standard method where the calibration curves ranged from 1 to 16 mg/mL of reference compounds linamarin, linustatin, and neolinustation using Model 2600 chromatography software, revision 3.1 (Nelson Analytical, Inc., Cupertino, CA). Linamarin was purchased from Calbiochem Corp. (La Jolla, CA). Linustatin and neolinustatin, fractionated from an aqueous ethanolic extract of linseed meal (Smith et al., 1980), were kindly provided by Dr. Palmer, South Dakota State University (Brookings, SD).

Statistical Analyses. The HCN contents in the HPLC determination were calculated stoichiometrically from the values obtained for the linamarin, linustatin, and neolinustatin concentrations. Analyses of variance by the general linear models (GLM) procedure, means comparison by Duncan's test, and variance components were performed according to SAS methods (SAS Institute, Inc., 1990).

RESULTS AND DISCUSSION

Method reproducibility is shown in Table I. Flaxseed was extracted in quadruplicate or quintuplicate with

0021-8561/92/1440-1346$03.00/0 Published 1992 by the American Chemical Society

Table I. Reproducibility of Linustatin and Neolinustatin Analysis under Reversed-Phase Conditions

cultivar/diglucoside	cyanogenic diglucosides,[a] mg/100 g, for extract					statistics		
	1	2	3	4	5	mean	SD	% RSD[b]
Linott								
linustatin	247	239	236	262	252	247	10.5	4.2
neolinustatin	186	171	166	189	181	178	9.8	5.5
Somme								
linustatin	323	350	347	340		340	12.2	3.6
neolinustatin	166	179	178	158		170	9.9	5.8

[a] Values for each extract are an average of two injections. [b] Percent relative standard deviation.

Table II. Cyanogenic Glucoside Content of Flax Cultivars[a]

cultivar	cyanogenic glucosides, mg/100 g of seed			
	linamarin	linustatin	neolinustatin	total
Andro	16.7 ± 3.8^{cd}	342 ± 38^{a}	203 ± 24^{a}	550 ± 53^{a}
Flanders	13.8 ± 3.7^{d}	282 ± 55^{c}	147 ± 22^{bc}	432 ± 47^{cde}
AC Linora	19.8 ± 5.4^{bcd}	269 ± 28^{c}	122 ± 20^{d}	402 ± 51^{def}
Linott	22.3 ± 8.2^{bcd}	213 ± 29^{e}	161 ± 25^{b}	396 ± 54^{ef}
McGregor	25.5 ± 4.0^{abc}	352 ± 56^{a}	91 ± 19^{e}	464 ± 76^{bc}
Noralta	20.3 ± 3.4^{bcd}	271 ± 34^{c}	163 ± 18^{b}	455 ± 50^{bcd}
NorLin	ND	295 ± 46^{bc}	201 ± 37^{a}	496 ± 81^{b}
NorMan	ND	231 ± 63de	135 ± 37^{cd}	365 ± 97^{f}
Somme	27.5 ± 12.1^{ab}	322 ± 46^{ab}	149 ± 25^{bc}	489 ± 78^{b}
Vimy	31.9 ± 8.4^{a}	262 ± 31^{cd}	115 ± 21^{d}	409 ± 54^{cdef}

[a] Means ± SD in a column followed by the same letter are not significantly different by Duncan's multiple range test at 5% level. Each value is the mean of six samples (duplicate injections of three extractions) analyzed individually. ND, not detected.

duplicate injections of each extract. Replicate extractions had a relative standard deviation (RSD) of 4.2% or less for linustatin and 6% or less for neolinustatin. The RSD between injections (n = 2) for any extract was 2% or less with few exceptions but was never greater than 5%. Linamarin, present in very low concentration, had a RSD of 12%.

The relative amounts of the three cyanogenic glucosides present in flaxseed differed significantly among cultivars (Table II). Linustatin was the major component of flaxseed, accounting for 54–76% of the total cyanogenic glucoside content. Its concentration in the 10 cultivars analyzed varied between 213 and 352 mg/100 g. The content of neolinustatin ranged from 91 mg/100 g for McGregor seed to 203 mg/100 g for Andro flaxseed, and, like linustatin, it was present in all samples analyzed. Schilcher and Wilken-Sauter (1986) reported a higher range for linustatin (218–538 mg/100 g) and neolinustatin (73–454 mg/100 g) in 49 samples of flax from Germany but detected only traces of linamarin. The monoglucoside linamarin was detected in eight cultivars and was absent in two cultivars, NorLin and NorMan. The content of linamarin in seed of the eight cultivars in which it was present ranged between 13.8 and 31.9 mg/100 g. The amount of linamarin detected in our samples may be due to a number of factors including extraction and analytical procedures, genotype of the material, as well as environmental factors.

Comparison of data in Table II indicates that often cultivars with a high concentration of linustatin are those associated with a high neolinustatin content. This was evident from a multicomparison analysis of the data (Table III). Nonsignificant correlation coefficients between linamarin, linustatin, and neolinustatin suggest that the contents of the three glucosides are independent of one another. However, this analysis also showed a highly significant positive relationship (r = 0.88) between linustatin and total cyanogenic glucoside content such that the highest glucoside cultivar showed the greatest linustatin content. The diglucosides, linustatin and neolinustatin, showed the highest correlation with regard to the effect

Table III. Correlation Coefficients for Cyanogenic Glucosides and Total Glucosides of Flaxseed

	linustatin	neolinustatin	total glucosides
linamarin	0.253	−0.127	0.255
linustatin		0.280[a]	0.882[b]
neolinustatin			0.636[b]

[a] $P < 0.0015$ (n = 126, except for linamarin where n = 71). [b] $P <$ 0.0001.

Table IV. Analysis of Variance for Cyanogenic Glucosides of Flax Grown at Three Locations

source	df	mean squares			
		linamarin	linustatin	neolinustatin	total
location (L)	2	0.73[b]	3.29	14.62[c]	25.32[b]
		(0.04)	(4.29)	(1.12)	(0)
cultivar (C)	2	0.94[c]	65.31[c]	15.18[c]	11.98
		(0.08)	(0)	(1.07)	(0.25)
L × C	4	0.22	13.82[c]	1.73[a]	22.30[b]
		(0.04)	(2.03)	(0.29)	(3.67)
error	27	0.08	2.18	0.57	4.18
CV %		15.28	8.11	7.56	6.85

[a–c] Significant at the 0.05, 0.01, and 0.001 probability levels, respectively. Values in parentheses are variance components.

that each glucoside had on the total glucosides, while linamarin, because of its small proportion, had less influence.

To further elucidate the cause of variation in flax cyanogenic glucosides, environmental effects (year and location) were studied in combination with cultivars. The results of analysis of variance for glucoside content of flax grown at three locations (Table IV) showed that the three glucosides were dependent on cultivar and location and generally there was cultivar × location interaction. Exceptions were for linustatin × locations and total glucosides × cultivars, indicating that linustatin content of flax was highly cultivar specific, although the total glucosides of the three cultivars studied did not differ significantly. Cultivar effect had a much larger relative contribution to the variation in linustatin levels than location or location × cultivar interaction. The variance components (Snedecor and Cochran, 1967) for cutivar location and location × cultivar interaction effects on linustatin were 4.3, 0, and 2.0, respectively. The variation in total glucosides was due to location and location × cultivar effects and none to cultivar effects. However, the cultivar–location variability does not play a significant role in the overall variability of the cyanogenic glucosides since its variance was generally smaller than that of the experimental error.

For the determination of seasonal effects, 10 flax cultivars grown at one location, Portage la Prairie, for 3 years (1987–1989) were analyzed. The results, summarized in Table V, show that cultivar, year, and their interactions were significant ($P < 0.01$) sources of variation for the individual as well as the total cyanogenic glucosides. Nonetheless, cultivar effects had a much larger relative contribution to the variation in the glucoside levels, especially neolinustatin, than year or year × cultivar interaction. The variance components for cultivar, year,

Table V. Analysis of Variance for Cyanogenic Glucoside of Flax Grown at Portage la Prairie in 1987–1989

source	df	mean squares			
		linamarin	linustatin	neolinustatin	total
year (Y)	2	2.54[b]	113.38[c]	7.62[b]	198.34[c]
		(0.05)	(2.39)	(0)	(3.84)
cultivar (C)	9 (7)[a]	2.82[c]	109.98[c]	64.25[c]	159.32[c]
		(0.15)	(6.82)	(4.33)	(7.83)
Y × C	18 (8)[a]	1.48[b]	26.45[c]	10.22[c]	66.31[c]
		(0.26)	(5.71)	(2.20)	(14.16)
error	96 (54)[a]	0.41	2.38	1.04	6.72
CV %		24.75	8.31	10.81	8.79

[a] Since cultivars NorLin and NorMan were devoid of linamarin, the degrees of freedom adjusted according to the ANOVA were 7, 8, and 54 for cultivar, year × cultivar, and error, respectively. [b] Significant at the 0.01 and 0.001 probability levels, respectively. Values in parentheses are variance components.

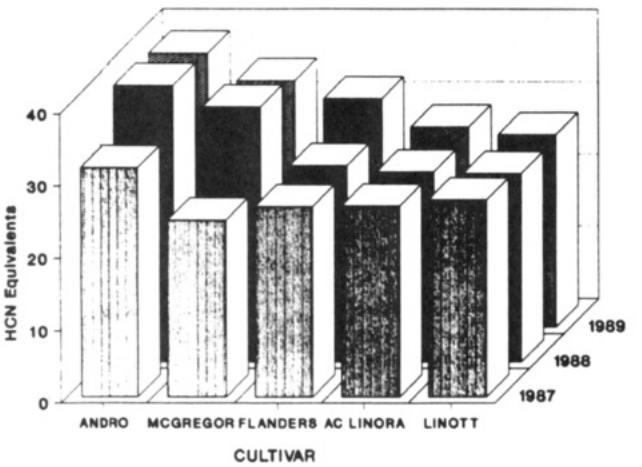

Figure 1. Total cyanogenic glucoside concentrations of five Canadian flax cultivars grown in three different years.

and year × cultivar interaction effects on neolinustatin were 4.3, 0, and 2.2, respectively. The variation in total glucosides and linustatin was mainly due to year × cultivar interaction and to a lesser extent to cultivar effects. Since the cultivar and cultivar × year variances are considerably larger than the experimental error, the cultivar and cultivar × year interaction play an important part in the overall variability of the cyanogenic glucosides.

The 1987–1989 growing seasons were particularly different in level of precipitation. The precipitation, especially during the green seedling and seed maturation stages of flax (July and August), in 1987 was 204 mm, double that of 1988 or 1989. The total glucosides of flax cultivars grown in 1988 and 1989 were higher than those of 1987, due largely to the contribution of the major diglucoside, linustatin. The cultivars grown in 1987 had low total glucosides (Figure 1), linustatin (Figure 2), and neolinustatin but high levels of linamarin. These results are consistent with those of Bhatty (1990, personal communication), who also reported an increase of about 70% in total glucosides in flax grown in 1989 in comparison to samples from the 1987 growing season. Cultivars grown in 1988 and 1989 differed only in the levels of linustatin and its related monoglucoside linamarin. The amount of linamarin stored in the endosperm of rubber (*Hevea brasiliensis*) reflected by its mobilization and utilization has been shown to proceed via a special pathway, where the related cyanogenic diglucoside linustatin acts as a transport form for the cyanogenic monoglucoside (Selmar et al., 1988). A similar situation possibly exists in the cyanogenic system of flax since both flax and rubber have the same developmental profile (Poulton, 1988). It is, therefore, possible to deduce by analogy that, in flax, drought conditions (i.e., moisture stress) either inhibit the mobilization of linamarin or increase its rate of utilization, thereby resulting in an increase in the level of linustatin. This in turn is reflected in an increase in total glucosides of flax.

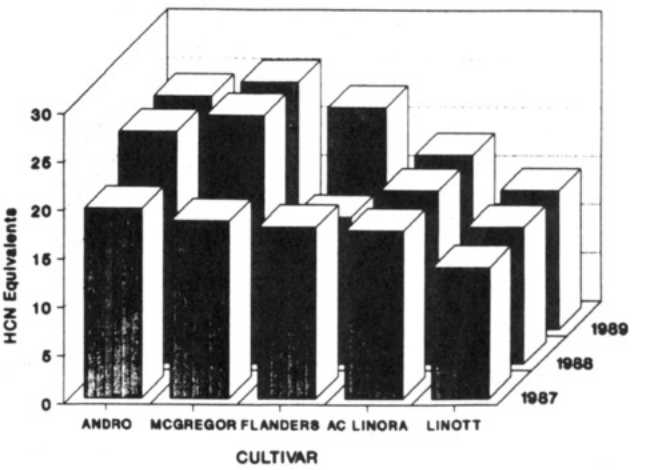

Figure 2. Linustatin concentration of five Canadian flax cultivars grown in three different years.

CONCLUSIONS

HPLC analysis of the cyanogenic glucosides in Canadian flax has revealed that the most abundant and common HCN-containing compounds of flaxseed are the diglucoside linustatin and neolinustatin. Linamarin, reportedly the main source of biologically bound HCN in flaxseed, was present at very low levels in 8 of the 10 cultivars analyzed. The content of all three cyanogenic glucosides was dependent on cultivar, location, and year of production of the seed, with cultivar being the most important factor. Thus, reduction in the content of cyanogenic glucosides in flaxseed may be possible through selection of low-cyanogenic glucoside genotypes.

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Received for review February 4, 1992. Revised manuscript received April 27, 1992. Accepted May 15, 1992.

Registry No. Linustatin, 72229-40-4; neolinustatin, 72229-42-6; linamarin, 554-35-8.