Determination of Cyanogenic Glycosides in Flaxseed by Barbituric Acid−Pyridine, Pyridine−Pyrazolone, and High-Performance Liquid Chromatography Methods†

M. Kobaisy,‡ B. D. Oomah,§ and G. Mazza*,‡

Food Research Program, Pacific Agri-Food Research Centre, Summerland, British Columbia V0H 1Z0, Canada, and Agri-Food Diversification Research Centre, Agriculture and Agri-Food Canada, Morden, Manitoba R6M 1Y5, Canada

A crude enzyme extracted from flaxseed by acetone precipitation was used for the hydrolysis of cyanogenic glycosides to determine total cyanide in flaxseed and flaxseed-derived products. The hydrolysis of cyanogenic glycosides (linamarin, linustatin, and neolinustatin) as well as the endogenous substrate was dependent on the crude enzyme concentration and followed a first-order relationship. Two colorimetric methods using the crude enzyme extract and an established high-performance liquid chromatographic (HPLC) method were compared using 28 flaxseed samples. Total HCN values obtained by all three methods were not statistically different, although those obtained by the HPLC method were higher than those from the colorimetric methods.

Keywords: *Flaxseed; cyanogenic glycosides; method comparison; crude enzyme extract; Linum usitatissimum; barbituric acid−pyridine; HPLC*

INTRODUCTION

The benefits of flaxseed in human nutrition have been known for a long time and have recently been reviewed (Cunnane and Thompson, 1995). The interest in phytochemicals and functional foods (i.e., foods that have alleged positive health benefits beyond inherent basic nutrition) has increased the use of flaxseed in some food products, or at least increased its awareness. However, flaxseed contains cyanogenic glycosides which release toxic hydrogen cyanide upon hydrolysis. Various procedures have been investigated to effect the breakdown of cyanogenic glycoside and detection of the HCN released in flaxseed (Mazza and Oomah, 1995). These include (1) acid hydrolysis followed by colorimetric assay of the dye complex formed with barbituric acid−pyridine (Bhatty, 1993), (2) the modified picrate method using β-glucuronidase for enzymatic hydrolysis and densitometry to measure the HCN formed (Brimer et al., 1983), and (3) analytical chromatographic methods such as gas−liquid chromatography (Kolodziejczyk and Fedec, 1995), thin-layer chromatography (Amarowicz et al., 1993), reverse-phase high-performance liquid chromatography (Oomah et al., 1992; Palmer, 1995; Schilcher and Wilkens-Sauter, 1986), and ion chromatography (Chadha et al., 1995).

Flaxseed also contains two cyanogenic β-glucosidases, linamarase and linustatinase (Fan and Conn, 1985), which are involved in the two-step catalytic hydrolysis of the cyanogenic glycosides to liberate HCN. Both linustatinase, which catalyzes the removal of the terminal glucose unit of linustatin forming glucose and the related monoglycoside linamarin, and linamarase were purified to homogeneity from flaxseed and characterized (Fan and Conn, 1985). Linustatinase hydrolyzes linustatin (relative velocity 100) and neolinustatin, a second cyanogenic glycoside present in flaxseed, with a relative rate of 59 but only slowly hydrolyzes linamarin (Conn, 1994). Linamarase is essentially inactive on linustatin (relative rate 1.3) and neolinustatin (relative rate 4.5) but, as expected, completely hydrolyzes linamarin (Fan and Conn, 1985).

Since flaxseed and products derived from it are increasingly being promoted for food and feed industries, there is an obvious need for more efficient analytical methods to detect cyanide in flaxseed and flax-based foods as a quality assurance measure and for "voluntary" or "optional" information on food labels dealing with phytochemicals. This study involved the extraction of crude enzyme from flaxseed and its use as a hydrolytic reagent in the determination of total cyanide in flaxseed and flaxseed products. In addition, two different colorimetric methods were compared to values obtained by an HPLC method.

MATERIALS AND METHODS

Flaxseeds from a previous study (Oomah et al., 1994), varying widely in cyanogenic glycoside content, were used. Flaxseed gum was extracted as described by Cui et al. (1994) by stirring flaxseed with distilled water (1:13, w/v) at 75 °C for 3 h. The extract was separated from the seeds with a 40 mesh screen and freeze-dried. Ethanol-extracted ground flax, flax pressed-cake, spent flaxseed, and CO_2-extracted flaxseed were kindly provided by Prof. D. A. Jenkins of the Department of Nutritional Science, University of Toronto (ON, Canada). These samples have been fully described (Jenkins, 1995).

All standard compounds were purchased from Sigma Chemical Co. (St. Louis, MO), except for linustatin and neolinustatin, obtained from Dr. I. S. Palmer (South Dakota State University, Brookings, SD).

Extraction of Enzyme from Flaxseed. Crude enzyme used in the assay of total cyanogens was extracted from flaxseed (cv. NorMan) ground with 5 vol of cold acetone (−20 °C) in a Waring blender for 1 min as described by Fan and Conn (1985). The slurry was filtered under vacuum through a Whatman No. 1 paper and the residue re-extracted three additional times with acetone. The resulting residue from the acetone extracts was placed in a desiccator under vacuum at 4 °C to remove the remaining trace of acetone. The extract

† Pacific Agri-Food Research Centre Contribution 956.

* Author to whom correspondence should be addressed [fax, (604) 494-0755; internet, MAZZAG@AGR.CA].

‡ Pacific Agri-Food Research Centre.

§ Agri-Food Diversification Research Centre.

S0021-8561(95)00838-7 CCC: $12.00 Published 1996 by the American Chemical Society

was homogenized with 8 parts (w/v) of cold sodium acetate buffer, pH 6, in a Waring blender for 1 min. The homogenate was centrifuged at 12000*g* for 2 h, and the supernatant was left in the fumehood at room temperature for 3 h to allow hydrolysis of any residual cyanogenic glycosides to HCN. The extract was then freeze-dried to remove the formed HCN and concentrated. The enzyme was stored at −20 °C and is referred to as crude enzyme extract.

Determination of Total Cyanide. Determination of total cyanide using barbituric acid−pyridine reagent was performed by a modification of the procedure described by Nambisan and Sundaresan (1984). An aliquot (0.1 mL) of flaxseed extract placed in a stoppered Quickfit test tube was evaporated with a stream of nitrogen. Sodium acetate buffer (0.5 mL, 0.1 M, pH 6) was added to the tube and the solution incubated with 0.5 mL of the crude enzyme extract for 1 h at 30 °C. The reaction was stopped by the addition of 1 mL of 0.2 M NaOH, allowed to stand for 5 min at room temperature, and neutralized with 0.2 M HCl. Chloramine T (1 mL, 0.5%, w/v) was added to 3 mL of buffered extract in a test tube followed by barbituric acid−pyridine reagent, prepared by dissolving 3 g of barbituric acid, 30 mL of pyridine, and 6 mL of concentrated HCl made to 100 mL with water. After allowing to stand at room temperature for 4 min, the absorbance of the resulting pink complex was measured at 585 nm in a Beckman Model DU 640 double-beam spectrophotometer.

Total cyanide was determined by a second colorimetric method using pyridine−pyrazolone reagent (Cooke, 1978; O'Brien et al., 1991). An aliquot of flaxseed extract (0.1 mL) was placed in a stoppered Quickfit test tube, evaporated with a stream of nitrogen gas, buffered with 0.5 mL of sodium phosphate buffer, 0.1 M, pH 6, mixed with crude enzyme extract (0.5 mL), and incubated at 30 °C. After 1 h the reaction was stopped by adding 0.6 mL of 0.1 M NaOH followed by sodium phosphate buffer (4.4 mL, 0.1 M, pH 6). The alkaline mixture ensured the total decomposition of the hydroxynitrites produced during enzymic cleavage of the cyanogenic glycosides. Chloramine T reagent (0.2 mL, 0.5%, w/v) was added to the buffered extract (3 mL) in a stoppered Quickfit test tube and mixed thoroughly. The tube was allowed to stand for 5 min in an ice−water bath, after which 0.8 mL of the pyridine−pyrazolone reagent (0.2%, w/v, in bis(pyrazolone) and 1% 3-methyl-1-phenyl-5-pyrazolone in pyridine) was added. The absorbance of the resulting blue complex was measured at 620 nm after 1 h. For both colorimetric methods, a cyanide calibration curve was prepared using potassium cyanide as a reference standard (concentration range 0.1−3.5 μg of cyanide (HCN)). Analyses were carried out in triplicate, and blanks containing the crude enzyme extract were run for each analysis.

Cyanogenic glycosides of flaxseed and products were analyzed using a high-performance liquid chromatograph (LKB, Pharmacia, Bromma, Sweden) equipped with a Lichrosorb RP-18 column, 10 mm particle size, 4 × 250 mm (Phenomenex, Torrance, CA), and a Shimadzu refractive index detector as described previously (Oomah et al., 1992). Total cyanide by the HPLC method was calculated stoichiometrically from values obtained for the cyanogenic glycoside (linamarin, linustatin, and neolinustatin) concentrations as described by Schilcher and Wilkens-Sauter (1986).

To determine total cyanide in flaxseed and flax products by the colorimetric methods described above, 100 mg of ground sample was extracted with aqueous ethanol (5 mL, 70%) in a sonic water bath for 30 min at 30 °C. The extract was filtered through glass wool and a 0.45 μm Millipore filter. A 100 μL aliquot of the extract was analyzed for total cyanide content.

At least three determinations were made for all assays. Comparison of the methods by *T*-test and correlation by the PROC CORR procedure were performed according to SAS methods (SAS, 1990).

RESULTS AND DISCUSSION

The activity of the crude enzyme extract was determined prior to its use for cyanide determination. Equimolar amounts (0.123 μmol) of the standards

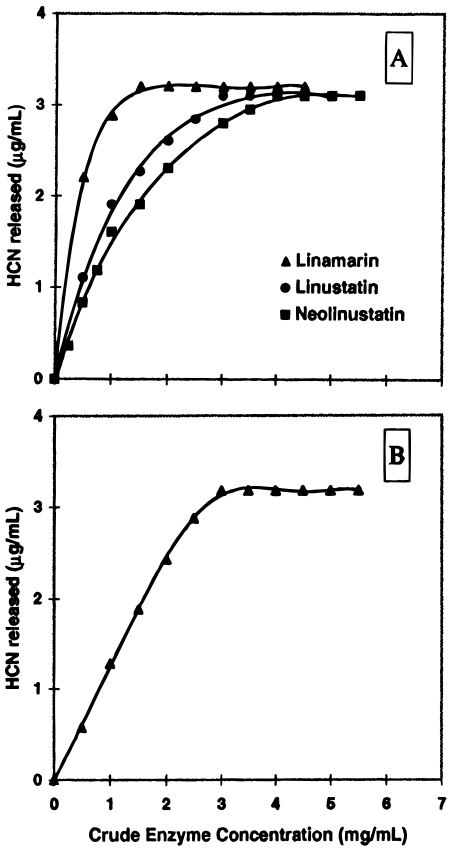

Figure 1. Effect of crude enzyme concentration on the hydrolysis of (A) cyanogenic glycoside standards and (B) endogenous substrate (flaxseed gum).

(linamarin, linustatin, and neolinustatin) were subjected individually to duplicate analyses for cyanide using different concentrations of the crude enzyme extract (Figure 1A). The enzyme incubation period was arbitrarily fixed (50 min) to minimize interference from any chemical or enzymatic side reactions that may occur. Total cyanide was estimated by the barbituric acid−pyridine (BAP) method. The minimum amount of enzyme required for complete recovery of linamarin, linustatin, and neolinustatin as cyanide was 1.5, 3, and 4 mg/mL, respectively. All cyanogenic constituents present in flaxseed (i.e. linamarin, linustatin, and neolinustatin) when incubated with the enzyme showed liberation of HCN. Such production of HCN was dependent on enzyme concentration (Figure 1A) and followed a first-order relationship:

$$R = R_{\max}(1 - e^{-KC})$$

where R is the amount of HCN released, $R_{\max}$ is the maximum HCN released at equilibrium, K is the rate constant, and C is the enzyme concentration. The release of HCN from linamarin was much faster than the hydrolysis of the diglycosides. The rate constants for linamarin, linustatin, and neolinustatin were 2.36, 0.84, and 0.65, respectively. These values reflect the reaction rate of the two enzymes linamarase and linustatinase present in the crude enzyme extract for the three endogenous substrates in flaxseed. The crude enzyme extract should have contained both linamarase and linustatinase activities since the procedure for its preparation was as described by Fan and Conn (1985). According to these authors, only 16 and 13% of linustatinase and linamarase enzymes, respectively, could be

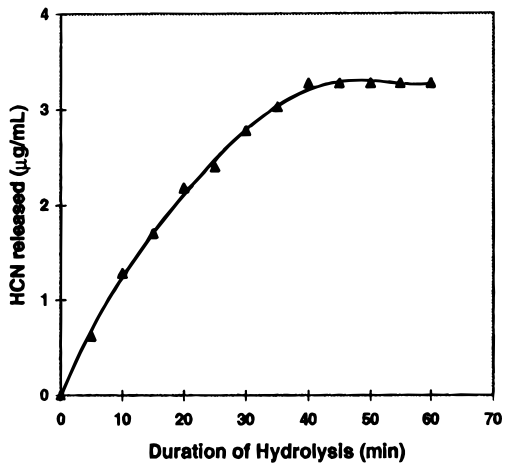

Figure 2. Time course hydrolysis of flaxseed gum.

recovered from the crude extract. Linustatinase hydrolyzes linustatin (relative velocity 100) and also neolinustatin with a relative rate of 59, while linamarase is essentially inactive on linustatin and neolinustatin (relative rates of 1.3 and 4.5, respectively) (Fan and Conn, 1985). The same extent of hydrolysis, i.e., linamarin > linustatin > neolinustatin was observed in this study (Figure 1A). The hydrolysis of a flaxseed gum extract incubated with different enzyme concentrations (Figure 1B) was similar to those of linustatin and neolinustatin.

Theoretically, 1 μmol of the cyanogenic glycoside yields 1 μmol of HCN. Recoveries from standards showed that 100% cyanide could be recovered from linamarin and 97% from linustatin and neolinustatin.

Furthermore, complete hydrolysis of linamarin, linustatin, and neolinustatin was also observed when an equimolar amount of the standards (0.123 μmol) was incubated individually with the crude enzyme extract (0.5 mL, 4 mg/mL) for 50 min using the pyridine−pyrazolone assay.

Ground cv. NorMan flaxseed was extracted for 30 min at 30 °C in 70% ethanol in a sonic water bath. Parts of the extract were spiked with linustatin, linamarin, and neolinustatin, respectively, and sonicated for another 5 min. The recoveries for each spiked sample were determined in triplicate with all matching assays and calculated as

$$\{[\mathrm{CNG}]_{\text{spiked extract}} - [\mathrm{CNG}]_{\text{extract}}\} = (\text{added CNG}) \times 100\%$$

[CNG] was evaluated from the A_{max} value of the KCN standard curve. The experiments showed a 94 ± 3% recovery of the cyanogenic glycosides, which indicates that there was no interference from the extract components on the quantitation of HCN in flaxseed samples. Similar recovery of linamarin, 90−95% with exogenous linamarase from cassava, was reported by Cooke (1978). The time required for complete hydrolysis of cyanogenic glycosides in flaxseed gum was determined by incubating the gum extract with the crude enzyme extract (0.5 mL, 4 mg/mL). A 40 min incubation time at 30 °C was sufficient to complete the reaction (Figure 2). Initially, for about 20 min, HCN was released rapidly as a result of strong cyanogenesis. HCN production continued thereafter but at a steadily decreasing rate until complete hydrolysis at 40 min. The HCN production potential at peak time (40 min) was equivalent to 3.25 μg of HCN/mL. Under the given conditions, the rate constant of the crude enzyme extract was 0.06 μg of HCN/mL/min based on a first-order reaction.

Table 1. Effect of Ethanol−H_2O Extraction Medium on Cyanogen Assay

flax seed cultivar	concentration of HCN (mg/100 g)		difference (%)
	no solvent	with solvent	
Tapeparana	0.27 ± 0.02	0.15 ± 0.02	56
Belinka	0.57 ± 0.02	0.37 ± 0.01	65
Linott	0.45 ± 0.02	0.27 ± 0.03	60
NorMan	0.31 ± 0.02	0.21 ± 0.02	68

Table 2. Comparison of the Three Methods for Cyanide Determination

	total cyanide (mg/100 g)		
	HPLC	pyridine−pyrazolone	barbituric acid−pyridine
mean (n = 28)	70.86	62.56	59.36
minimum	0.86	1.82	1.67
maximum	762.03	675.00	650.00
SE (% of mean)	37.41	37.37	36.27
method comparison			
difference between HPLC		8.30 (0.82)[a]	11.50 (0.74)
difference between pyride−pyrazolone			3.20 (0.92)[a]

[a] Data in parentheses are probability of T values.

In flaxseed samples extracted with 70% aqueous ethanol, no free hydrocyanic acid was detected; therefore evaporation of ethanol prior to analysis had no effect on the total cyanide recoveries. However, ethanol affects the enzyme activity, and its evaporation involves extra work. Experiments to determine whether the presence of ethanol affected the assay of cyanogens were carried out using flaxseed cultivars with a wide range of cyanogen contents. The results (Table 1) show that the effect of ethanol on the assay was highly significant (56−68%), although a large excess of enzyme extract (5 mg/mL) was used for these experiments. Concentrations of ethanol up to 25% level amounted only to a 1−3% change in cyanogen assay using cassava linamarase (O'Brien et al., 1991). The large difference observed may be due to the well-known biphasic response of β-glucosidase activity to alcohols (Glew et al., 1994) where competitive inhibition is observed at high concentrations of alcohols. Extraction with acidic solvents, such as 0.1 M orthophosphoric acid with or without 25% ethanol, resulted in poor recovery of cyanogens and required filtration and centrifugation due to the viscous nature of the extract. A similar occurrence was reported for the assay of cyanoglucosides in cassava (Nambisan and Sundaresan, 1984) except that complete extraction was achieved with an acidic solvent.

The three methods described earlier were compared using 28 samples of flaxseed ranging from 1 to 762 mg of total HCN/100 g of sample (Table 2). No statistical difference was found in the cyanogen content obtained by the methods. However, the HPLC and the BAP methods gave the highest and lowest average values, respectively. The low values of the colorimetric methods could be due to HCN loss in the reaction medium or to HCN bound to autolysis components (Harris et al., 1980). Our results do not agree with those of Schilcher and Wilkens-Sauter (1986), who found that the HCN concentrations determined using HPLC were about 2% lower on average than those determined photometrically. The HPLC method based on separation techniques is a direct method able to distinguish all three cyanogenic compounds present in flaxseed and is thus

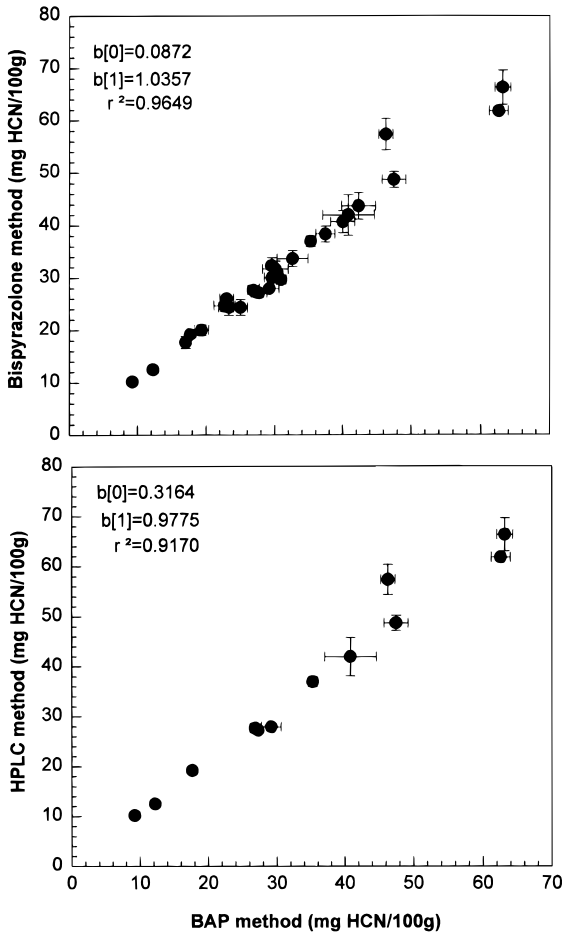

Figure 3. Relationship of total HCN values obtained by the bis(pyrazolone) and barbituric acid−pyridine methods (top) and HPLC and barbituric acid−pyridine methods (bottom).

likely to give more accurate cyanogen data. The colorimetric methods are indirect, not based on separation science, and hence suffer from low biases. The difference between the colorimetric methods was small. In spite of these differences, the accuracy and analytical error associated with these methods were consistent at about 37% standard error of the mean (Table 2). Results obtained by the three methods correlated highly ($r = 0.99$, $P = 0.0001$) with each other. Bhatty (1993), using chemical hydrolysis, also reported that the HCN content of seed from seven cultivars of flax determined by the barbituric acid−pyridine reagent was significantly correlated ($r = 0.96$) to total cyanogen determined by our HPLC method (Oomah et al., 1992). The close agreement of the BAP method with the other two methods is illustrated in Figure 3.

Although the HPLC method is most sensitive and accurate for the determination of cyanogenic glycosides in flaxseed, it is time consuming and involves the use of standards difficult to obtain. The BAP method is a quick alternative method for the determination of total cyanogen.

LITERATURE CITED

Amarowicz, R.; Wanasundara, P. K. J. P. D.; Shahidi, F. TLC separation of linamarin, linustatin and neolinustatin. *Nahrung* **1993**, *37*, 88−90.

Bhatty, R. S. Further compositional analyses of flax: mucilage, trypsin inhibitors and hydrocyanic acid. *J. Am. Oil Chem. Soc.* **1993**, *70*, 899−904.

Brimer, L.; Christensen, S. G.; Molgard, P.; Nartey, P. Determination of cyanogenic compounds by thin-layer chromatography. 1. A densitometric method for quantification of cyanogenic glycosides, employing enzyme preparations (β-glucuronidase) from Helix pomatia and picrate-impregnated ion-exchange sheets. *J. Agric. Food Chem.* **1983**, *31*, 78−793.

Chadha, R. K.; Lawrence, J. F.; Ratnayake, W. M. N. Ion chromatographic determination of cyanide released from flaxseed under autohydrolysis conditions. *Food Addit. Contam.* **1995**, *12*, 527−533.

Conn, E. E. β-glycosidases in plants: substrate specificity. In *β-glucosidases: Biochemistry and Molecular Biology*; Esen, A., Ed.; American Chemical Society: Washington, DC, 1994; pp 15−26.

Cooke, R. D. An enzymatic assay for the total cyanide content of cassava (*Manilot esculenta* Crantz). *J. Sci. Food Agric.* **1978**, *29*, 345−352.

Cui, W.; Mazza, G.; Oomah, B. D.; Biliaderis, C. G. Optimization of an aqueous extraction process for flaxseed gum by response surface methodology. *Lebensm.-Wiss. Technol.* **1994**, *27*, 363−369.

Cunnane, C. S.; Thompson, L. U. *Flaxseed in Human Nutrition*; AOCS Press: Champaign, IL, 1995; p 384.

Fan, T. W.-M.; Conn, E. E. Isolation and characterization of two cyanogenic β-glucosidases from flax seeds. *Arch. Biochem. Biophys.* **1985**, *243*, 361−373.

Glew, R. H.; Gopalan, V.; Forsyth, G. W.; VanderJagt, D. J. The mammalian cytosolic broad-specificity β-glucosidase. In *β-glucosidases: Biochemistry and Molecular Biology*; Esen., A., Ed.; American Chemical Society: Washington, DC, 1994; pp 83−112.

Harris, J. R.; Merson, G. H. J.; Hardy, M. J.; Curtis, D. J. Determination of cyanide in animal feeding stuffs. *Analyst* **1980**, *105*, 974−980.

Jenkins, D. J. A. Incorporation of flaxseed or flaxseed components into cereal foods. In *Flaxseed in Human Nutrition*; Cunnane, S. C., Thompson, L. U., Eds.; AOCS Press: Champaign, IL, 1995; pp 281−294.

Kolodziejczyk, P. P.; Fedec, P. Processing flaxseed for human consumption. In *Flaxseed in Human Nutrition*; Cunnane, S. C., Thompson, L. U., Eds.; AOCS Press: Champaign, IL, 1995; pp 261−280.

Mazza, G.; Oomah, B. D. Flaxseed, dietary fiber and cyanogens. In *Flaxseed in Human Nutrition*; Cunnane, S. C., Thompson, L. U., Eds.; AOCS Press: Champaign, IL, 1995; pp 56−81.

Nambisan, B.; Sundaresan, S. Spectrophotometric determination of cyanoglucosidases in cassava. *J. Assoc. Off. Anal. Chem.* **1984**, *67*, 631−643.

O'Brien, G. M.; Taylor, A. J.; Poulter, N. H. Improved enzymic assay for cyanogens in fresh and processed cassava. *J. Sci. Food Agric.* **1991**, *56*, 277−289.

Oomah, B. D.; Mazza, G.; Kenaschuk, E. O. Cyanogenic compounds in flaxseed. *J. Agric. Food Chem.* **1992**, *40*, 1346−1348.

Oomah, B. D.; Kenaschuk, E. O.; Mazza, G. Safety considerations of flaxseed and flaxseed components. In *Proceedings of the 55th Flax Institute of the United States*; Flax Institute: Fargo, ND, 1994; pp 178−182.

Palmer, I. S. Effects of flaxseed on selenium toxicity. In *Flaxseed in Human Nutrition*; Cunnane, S. C., Thompson, L. U., Eds.; AOCS Press: Champaign, IL, 1995; pp 165−173.

SAS Institute. *SAS/STAT User's Guide*, version 6, 4th ed.; SAS Institute: Cary, NC, 1990.

Schilcher, H.; Wilkens-Sauter, M. Quantitative determination of cyanogenic glycosides in *Linum usitatissimum* using HPLC. *Fette, Seife, Anstrichm.* **1986**, *8*, 287−290.

Received for review December 27, 1995. Revised manuscript received June 19, 1996. Accepted June 28, 1996.[⊗]

JF950838J

[⊗] Abstract published in *Advance ACS Abstracts,* September 1, 1996.