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Article

Determination of cyanide in whole seeds and brans of linseed (*Linum usitatissimum* Linn) by molecular spectrophotometry

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The addition of linseed (*Linum usitatissimum* Linn) in the diet, as a functional food, has increased over the years. However, it possesses cyanogenic glycosides. This study aimed to quantify and compare cyanide concentration in whole seed and bran of brown and golden types to establish a safe limit of intake. Three commercial labels, from brown and golden whole seed types (Ab, Ag, Bb, Bg, Cb and Cg), and six commercial labels of brown and golden bran (1b, 2g, 3g, 4b, 5g, and 6b), were selected, totalizing twelve samples. Total cyanide concentration was quantified by a colorimetric method employing alkaline picrate, after acid hydrolysis. The whole seed cyanide values were between 348.4 and 473.20 μg/g and the bran cyanide values were between 459.53 and 639.35 μg/g. The analyzed bran presented increased cyanide concentrations than the whole seeds with no differences between brown and golden types. Food able to produce cyanide less than 90 μg/kg body weight, daily, is considered secure for consumption. Considering this limit and analyzed samples, it is safe to eat approximately two tablespoons of seeds or one tablespoon of bran. These results point out the importance of cyanide amount daily intake information to be in linseed packaging, to ensure secure consumption.

Keywords: Linseed. Seed. Bran. Cyanide. Toxicity.

INTRODUCTION

The search for healthy eating habits has been increasing over the years, so the demand for functional foods grows in the same proportion. Thus, the presence of cereals, seeds, and brans are much more evident in the meals of people all around the world. However, some foods, besides having a high nutritional value, also have toxic non-nutritive agents, which under conditions of excessive ingestion, may pose risks to human health.

Linseed (*Linum usitatissimum* Linn), a plant from the Linaceae family, is an example of functional food with rising demand by population, in its various forms (seed, bran, oil), because of its important nutritional components, such as essential fatty acids, fiber and proteins (Rosling, 1993; Attia *et al.*, 2022abc; Shim *et al.*, 2022; Sirotkin, 2023). Linseed protein have been reported to possess anti-diabetic, anti-inflammatory, antihypertensive and antioxidative properties (Dzuvor *et al.*, 2018; Keykhasalar *et al.*, 2021; Attia *et al.*, 2022abc; Mueed *et al.*, 2022ab; Sirotkin, 2023). What is not known by population, however, is that, in addition to these healthy nutritional compounds, linseed contain the cyanogenic glycosides linamarin, linustatin, neolinustatin and lotaustralin (Shim *et al.*, 2014; Abraham, Buhrke, Lampen, 2016; Mueed *et al.*, 2022ab), which can be harmful to human health.

Cyanogenic glycosides (CG) are organic compounds with defense function, produced by plants. Consist of a

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sugar (glycone) moiety and a non-sugar (aglycone) moiety. Because they are water-soluble, when hydrolyzed they release cyanide (CN) or hydrocyanic acid (HCN), present in its aglycone portion (Abraham, Buhrke, Lampen, 2016; Mueed *et al*., 2022ab). This hydrolysis reaction in fresh vegetables occurs through enzymes, when injured, or during preparation for consumption, being favored in acid medium. The CG molecules in the gut are hydrolyzed by gastrointestinal β-glycosidases or by the digestive tract acidic environment releasing HCN. The hydrolysis of linustatin releases linamarin and β-glucose. Linamarin hydrolysis products are glucose, propane and HCN, as illustrated in Figure 1 (Midio, Martins, 2000; Schwarz, Martins, 2016; Mueed *et al*., 2022ab).

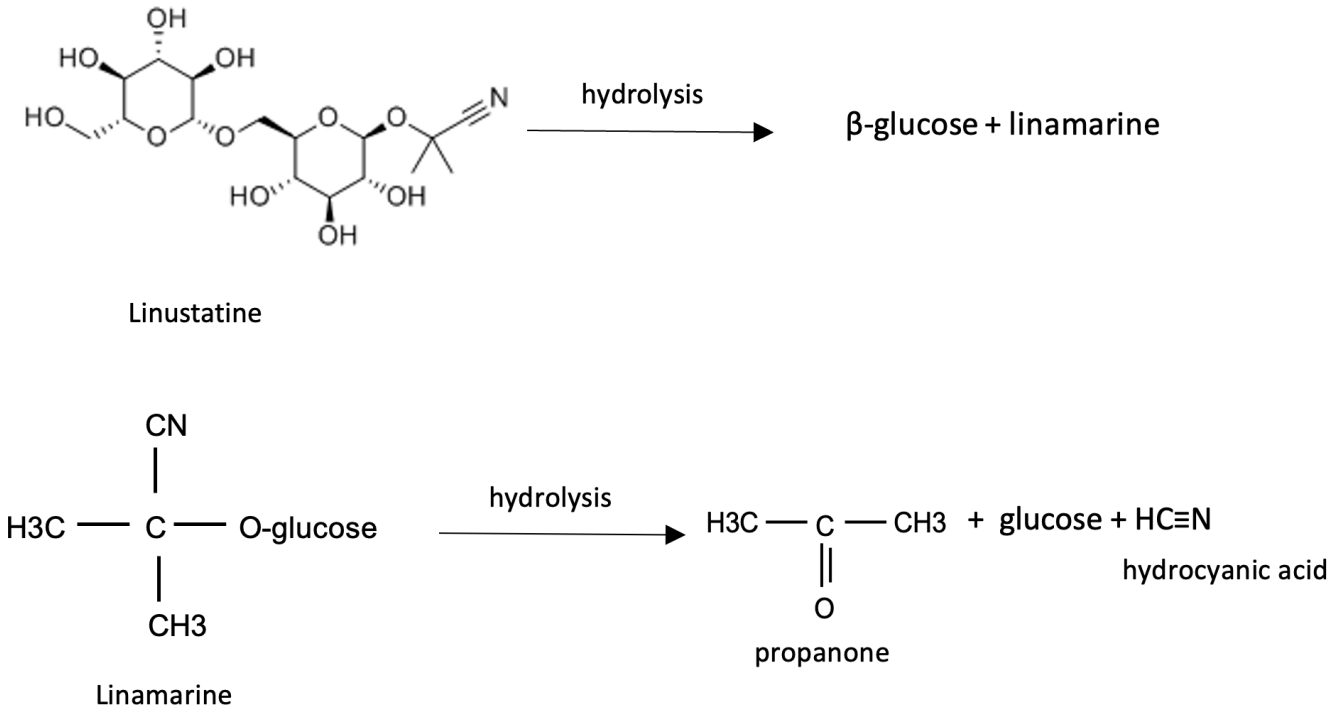

FIGURE 1 – The hydrolysis of the cyanogenic glycosides linustatin and linamarin, found in linseed.

Cyanide (CN), a respiratory inhibitor, poses a risk of poisoning as it has great affinity for the trivalent iron of the mitochondrial respiratory chain cytochrome oxidase (cytochrome a3) enzyme. When cyanide binds to the cytochrome a3 enzyme, a stable complex is formed that prevents electron transport between the enzyme and molecular oxygen, resulting in inhibition or reduction of oxidative metabolism and phosphorylation, culminating in cytotoxic hypoxia (Midio, Martins, 2000; Schwarz, Martins, 2016; Zuk *et al*., 2020). The decrease in oxygen is perceived by chemoreceptor cells that, by feedback, stimulate the increase in respiratory rate. A transient state of central nervous system stimulation is observed leading to headache, nausea, nervousness, confusion, rapid heartbeat, hypoxic seizures, discoloration of the skin as a result of oxygen insufficiency and, in extreme cases, death due to central respiratory system failure, characterizing acute intoxication (Cressey, Reeve, 2019; Midio, Martins, 2000; Morrison, 2019; Zuk *et al*., 2020).

Both HCN and CN are absorbed and then can be converted to thiocyanates by the mitochondrial enzyme rhodanese. Chronically, thiocyanates disrupt iodine uptake from the thyroid gland and promote iodine-deficiency illness such as goiter, characterizing chronic intoxication (Bekhit *et al*., 2018).

As CN detoxification pathways, thiosulfate and sulfur transferase convert about 70% of CN to thiocyanate, acting as sulfur donors for the rhodanese and sulfotransferase enzymes (Mueed *et al*., 2022ab). The availability of sulfur appears to be a rate-limiting step in the detoxification of cyanide. Around 15-20% of total CN metabolism depends on the reaction between CN and L-cystine, resulting in the formation of 2-amino-2-thiazoline-4-carboxylic acid. It is also known that CN binds to methemoglobin and hydroxocobalamin, forming compounds that are eliminated in the urine (Schrenk *et al*., 2019).

Food toxicology is intended to recognize possible toxic substances in foods to establish safe forms and secure quantities for consumption. The presence of CG in linseed is neglected in commercial packages at markets. This work aimed to quantify and compare total cyanide present in golden and brown types of whole seeds and bran, by molecular absorption spectrophotometry, to help establish a safe limit of intake and propose a secure way of consumption by adding cyanide content information in packages, as nutritional facts.

MATERIAL AND METHODS

Glassware, consumables, and equipment

105° angle glass adapter, Bel M6202 semi-analytical balance, Solab SL 150/22 water bath, round bottom glass flask (1000 mL), volumetric flask (25 mL, 50 mL and 100 mL), glass rod, glass beaker (50 mL and 100 mL), straight condenser, glass graduated Erlenmeyer (125 mL), visible SP molecular absorption spectrophotometer -1105, thread sealant, separating glass funnel (250 mL), glass funnel, Quimis Q.321.A.25 heating blanket, disposable polyethylene Pasteur pipette 2 mL, graduated glass pipette (1 mL, 2 mL, 5 mL and 20 mL), graduated glass beaker (50 mL), plastic graduated beaker (100 mL and 250 mL).

Reagents

Picric acid P.A Cinética®, sulfuric acid P.A Vetec®, distilled water, sodium carbonate P.A Vetec®, sodium cyanide P.A Vetec® and sodium hydroxide P.A Vetec®.

Samples

The whole seeds and bran samples analyzed were acquired in Natal/RN main supermarkets and natural products stores, in 2017, from September to October. For the whole seeds, three trademarks (A, B and C) of brown (b) and golden (g) types were selected, totaling six samples, which were named Ag, Ab, Bg, Bb, Cg, and Cb. For the brans, brown (b) and gold (g), trademarks were selected, being 3 samples of each type, totaling six samples, which were named 1b, 2g, 3g, 4b, 5g, and 6b.

An aliquot of 20 g of each sample was weighed individually on a semi-analytical centesimal scale. Each sample was analyzed in duplicate in the Toxicology Laboratory of the Faculty of Pharmacy – UFRN by the methodology described by William (1984) and adapted by Schwarz and Martins (2016).

Acid Hydrolysis

Each sample aliquot (20 g) was transferred individually into a round bottom flask coupled to a distiller. The distillation system was closed by dipping the end of the condenser into an Erlenmeyer containing 20 mL of sodium hydroxide (NaOH) solution (2.5%). Then, 80 mL of distilled water and 20 mL of sulfuric acid (10%) were added, initiating hydrolysis. A three-hour acidic hydrolysis time was standardized for each sample.

Distillation

At the end of the three-hour hydrolysis, 40 mL of sulfuric acid (10%) was added, initiating distillation. Heat catalyzes the breakdown of glycosidic bonds and releases HCN. HCN is volatile and flows into the Erlenmeyer containing sodium hydroxide (2.5%) solution.

In a first distillation, 125 ml of distillate, called Distillate 1 (D1), was collected. A second distillation was performed after replacing the Erlenmeyer located at the end of the condenser with another, also initially containing 20 mL of sodium hydroxide (2.5%) solution. Before the beginning of this second distillation, 80 mL of distilled water and 20 mL of 10% H_2SO_4 were added. Then, 125 mL of distillate, Distillate 2 (D2), was collected.

Colorimetric Determination

A 5 mL aliquot of each distillate was transferred to tubes. In each test tube, 5 mL of alkaline picrate solution (0.5%) was added. In a third tube (reagent blank) was added 5 mL of distilled water and 5 mL of alkaline picrate solution (0.5%). The tubes were shaken, closed and taken to a water bath at 70° C for ten minutes. Its absorbances were measured at 490 nm.

Calibration Curves

For the construction of the calibration curves, a sodium cyanide solution (50 µg/mL) was used. Aliquots of 1.0, 2.0, 3.0, 4.0 and 5.0 mL of this solution were transferred to tubes. The volume of each tube was made up to 5 mL with distilled water and 5 mL of alkaline picrate solution (0.5%), corresponding to 50, 100, 150, 200 and 250 µg CN. The tubes were taken to a water bath at 70° C for ten minutes. After cooling, the absorbances of the standard solutions were measured at 490 nm.

Calculations

CN concentration (in µg/mL) in distilled 1 and 2, for each sample, were calculated employing the equation of the calibration curves. The CN concentration mean was used for each sample, analyzed in duplicate. The total CN concentration found in each sample was obtained in µg/g (ppm) by the following equation:

µg CN/g sample = (m x D)/(p x v) where:
m - amount (in µg) of CN in the distillate aliquot
D - the total volume of distillate
p - the weight (g) of the sample used for analysis
v - the volume of distillate used for analysis

Statistical analyses

The CN concentration of whole seeds and brans were analyzed by the Student t test. The one-way ANOVA post-hoc test was applied, when necessary. The difference of 5% was adopted and the level of significance was set at $p<.05$. The software GraphPad Prism 8.0.2 (California/USA, 2017) was used.

RESULTS

The samples were analyzed in duplicate. The mean absorbance of distilled 1 and distilled 2 and the mean CN concentration, of each sample, are presented in Tables I and II. The whole seed samples revealed CN concentrations between 348.4 ppm and 473.20 ppm (mean = 415.40 µg/g), as shown in Table I. The six bran samples revealed CN concentration between 459.53 µg/g and 639.35 µg/g (mean= 529.40 µg/g), as shown in Table II. The Student t test [$t=2.951$; $F(1,11)=10$; $p=.0145$] revealed statistical differences between whole seeds and brans CN concentration, with 114.00 µg/g ± 38.62 (difference between means ± standard error deviation). The one-way ANOVA showed increased CN concentration in the bran samples when compared to whole seed samples ($p<.05$*). No CN concentration differences were found between brown and golden types of whole seeds and brans.

TABLE I – Absorbance and amount of CN (µg) means in distillates 1 and 2 and total CN concentration (µg/g) in whole seeds of golden and brown linseed types

Sample	Mean D1 absorbance	Mean D1 CN (µg/mL)	Mean D2 absorbance	Mean D2 CN (µg/mL)	Total CN (µg/g)
Ag	1.297	133.58	1.175	214.83	348.40
Ab	1.053	167.40	1.300	201.83	369.27

TABLE I – Absorbance and amount of CN (µg) means in distillates 1 and 2 and total CN concentration (µg/g) in whole seeds of golden and brown linseed types

Sample	Mean D1 absorbance	Mean D1 CN (µg/mL)	Mean D2 absorbance	Mean D2 CN (µg/mL)	Total CN (µg/g)
Bg	0.773	168.99	1.105	249.17	418.17
Bb	0.929	145.84	1.516	270.17	416.01
Cg	0.938	194.28	1.312	278.92	473.20
Cb	0.922	204.69	1.138	262.84	467.54

D = Distilled; A, B, C = brands; g=golden linseed type; b=brown linseed type. Calibration curves: Ag (y=0,004X + 0,193; r^2= 0,957), Ab (y=0,007X + 0,092; r^2= 0,994), Bg (y=0,004X + 0,136; r^2= 0,985), Bb (y=0,005X + 0,252; r^2= 0,982), Cg (y=0,005X + 0,160; r^2= 0,980), Cb (y=0,005X + 0,103; r^2= 0,980).

TABLE II – Absorbance and amount of CN (µg) means in distillates 1 and 2 and total CN concentration (µg/g) in bran samples of golden and brown linseed types

Sample	Mean D1 absorbance	Mean D1 CN (µg/mL)	Mean D2 absorbance	Mean D2 CN (µg/mL)	Total CN (µg/g)
1b	1.360	255.99	0.716	118.96	468.67
2g	1.500	267.38	0.880	244.10	639.35
3g	1.156	286.98	0.887	134.04	485.51
4b	1.422	217.84	1.318	165.10	459.53
5g	1.071	277.02	1.217	220.96	622.48
6b	1.406	186.00	1.321	214.63	500.80

D = Distilled; 1,2,3,4,5,6 = brands; b= brown linseed type; g = golden linseed type. Calibration curves: 1b (y=0,004X + 0,156; r^2= 0,973), 2g (y=0,005X + 0,122; r^2= 0,987), 3g (y=0,005X + 0,045; r^2= 0,987), 4b (y=0,005X + 0,146; r^2= 0,975), 5g (y=0,005X + 0,16022; r^2= 0,978), 6b (y=0,004X + 0,124; r^2= 0,994).

DISCUSSION

The methodology employed, acid hydrolysis followed by complexation with alkaline picrate is considered effective in the determination of total cyanide in foods (William, 1984; Bradbury, 2009). The HCN released reacts with NaOH. The product formed, sodium cyanide (NaCN), is stable, making it possible to quantify CN in the sample. Alkaline picrate is reduced by CN to form a red-orange compound, with absorbance measured by the spectrophotometer at 490 nm. Molecular absorption spectrophotometry was used as a way of detection and quantification of total CG as, aforementioned, is the most widely employed method for this purpose (William, 1984; Bradbury, 2009; Tivana, 2014; Schwarz, Martins, 2016; Mueed *et al.*, 2022b). The total CG can be determined by hydrolyzing glycosides and measuring the amount of HCN released, besides measuring glycosides directly. This indirect method cannot determine specific glycoside concentrations, but it can calculate the amount of total glycosides present in a sample. Several authors have reported similar results using either the direct method

or indirect approaches (Bacala, Barthet, 2007; Yamashita *et al*., 2007).

According to Ikediobi, Onyia, Eleuwah (1980), vegetables or fractions with CN contents above 100 µg/g are considered toxic. Therefore, the samples analyzed in the current study can be classified as toxic, if considering the limit proposed above. JECFA (2012) currently proposes an acceptable daily intake (IDA) of 90 µg CN/kg body weight for food or its fractions that, once ingested, are capable of releasing CN. Thus, according to the results obtained from the samples analyzed, it is considered safe for a human being, with an average weight of 70 kg, to consume, daily, 18.08g of whole seeds that possess 348.4 µg CN/g, and 13.31g of seeds that possess 473.2 µg CN/g. For bran, it is considered safe for a human being, with an average weight of 70 kg, to consume daily 13.71g of bran containing 459.53 µg CN/g, and 9.85g of bran containing 639.35 µg CN/g.

If one full tablespoon of whole seeds weighs approximately 6 g, is possible establish as safe, according to the proposed IDA= 90 µg CN/kg body weight (JECFA, 2012), the daily ingestion of two tablespoons of the whole linseed containing the higher CN dose detected (473.20 ppm). Besides, if a full tablespoon is equivalent to a 15 g portion of linseed bran - according to the nutritional information contained in the packaging of the analyzed samples - is possible suggest as safe, considering the proposed IDA, the daily ingestion of less than one tablespoon of linseed bran containing the higher CN dose detected (639.35ppm). It is important to highlight that this suggestion considers the release of available CN in whole seeds and bran.

Considering the data obtained with the samples analyzed, linseed intake can be safe since it is added in small quantities to daily diet. In this study, the bran samples presented increased CN concentration than the whole seeds, as reported before (Cressey, Saunders, Goodmann, 2013). It is known that the lethal dose of CN to humans ranges from 0.5 to 3.5 mg/kg body weight (Wogan, Marletta, 1993; Mueed *et al*., 2022ab). Otherwise, people who consume more than two spoons per day do not show signs of intoxication possible by heating and cooking seeds or brans in diet, which can decrease the CN content (Safdar *et al*., 2020; Mueed *et al*., 2022ab). Also, the crushing of the whole seeds is partial during the chewing process, which can impair the release of an important cyanide fraction, decreasing its availability (Mueed *et al*., 2022ab).

CONCLUSION

The methodology employed found CN values between 348.40 and 467.54 µg/g in whole seeds and between 459.528 µg/g to 639.35 µg/g in brans. The brans samples presented increased cyanide levels than the whole seeds samples. Although there are many studies available on linseed and the knowledge of the presence of CG in its composition, few were found regarding the quantification of cyanide for a secure daily intake. Therefore, according to the safe daily intake of 90 µg cyanide/kg body weight, suggested by JECFA (2012), it can be considered safe to eat approximately two full tablespoons of whole seeds per day or one full tablespoon of bran per day, which can easily be exceeded in daily intake. Thus, the proposal to add the total CN content in commercial packages may ensure a secure consumption of this nutritious and healthy food, by the population.

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INTEREST CONFLICTS

There are no conflicts of interest by any of the authors.

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