ABSTRACT

Title of thesis: Evaluation of the Simple Wetting Method to Reduce Total Cyanogens in Flaxseed

Kathleen Suzanne Sanders, Masters of Nutrition and Food Science, 2016

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A simple wetting method used to significantly reduce the % total cyanide remaining (TCR) in cassava was evaluated for its applicability to flaxseed. An optimal simple wetting method (SWM) in terms of time, temperature, and exposed surface area (SA) depth was developed for reducing the % TCR in ground flaxseed. The optimal parameters were a treatment time of 12 h, an ambient temperature of approximately 22°C, and an increased exposed SA achieved through spreading the wetted flaxseed mixture out to a thin depth of 0.5 cm. General mixed model analysis confirmed that the variables time and SA were significant factors in minimizing the %TCR in the optimal model. Pairwise comparison showed that the 0.5 cm depth had the greatest effect. The desirable lignan secoisolariciresinol diglucoside (SDG) was significantly retained, as determined by HPLC quantification of pre- and post-processing levels.

EVALUATION OF THE SIMPLE WETTING METHOD TO REDUCE TOTAL CYANOGENS IN FLAXSEED

by

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Dedication

First and foremost I would like to thank my family members, especially my daughters Sandra and Janelle Winters, my son John Winters, and my brother Marvin Sanders for their support and encouragement in this endeavor. I am also grateful for the guidance of those long gone, but not forgotten; namely my father and grandparents. Your endurance and struggles encourage me still to fight a good fight and to continue forward. I love, respect, and admire you all…

I would also like to thank my friends and co-workers, especially Dr. David Rao, at the Food Components and Health Laboratory of the Beltsville Human Nutrition Research Center of the United States Department of Agriculture for their technical assistance and words of wisdom. I wish you all the best!

Finally I wish to thank my advisor, Dr. Robert Buchanan for guiding me in the right direction and for *strongly* suggesting that I keep it simple. Thank you also to Dr. Bradbury for demonstrating the value of simplicity, especially when combined with knowledge and shared through education.

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LIST OF ABBREVIATIONS

BHNRC	Beltsville Human Nutrition Research Center
CFU	Colony forming unit
FAO	Food and Agriculture Organization
GLMM	General linear mixed models
HCN	Hydrogen cyanide
HPLC	High-performance liquid chromatography
JEFCA	Joint FAO/WHO Expert Committee on Food Additives
LAB	Lactic acid bacteria
PA	Peak Area
PNPG	p-nitrophenyl-*B*-D-glucopyranoside
RH	Relative humidity
RSM	Response surface methodology
SA	Surface Area
SDG	Secoisolariciresinol diglucoside
SWM	Simple wetting method
TCC	Total cyanide content
TCR	Total cyanide remaining
USDA	United States Department of Agriculture
WHO	World Health Organization

Introduction

Flaxseed is an oilseed that conveys many health benefits including, but not limited to, a reduced risk of cardiovascular disease, diabetes, and hormone-sensitive cancers. The positive human health impact may be largely attributed to the extraordinarily high levels of alpha-linolenic acid and lignans, as well as the high quality proteins and soluble fiber found in flax (Muir &Westcott, 2003). Alpha-linolenic acid is an essential omega-3 fatty acid which constitutes about 57% of the total fatty acids in flaxseed and is a potent inhibitor of pro-inflammatory mediators (Oomah, 2001). Plant lignans are phenolic compounds that act as both antioxidants and phytoestrogens. Flaxseed contains up to 75 times more lignans than other known plant foods and is primarily composed of the lignan secoisolariciresinol diglucoside (SDG) (Axelson et al., 1982; Thompson et al., 1991). Lignans inhibit cell proliferation and growth, and flaxseed SDG has shown promising effects in reducing the growth of hormone sensitive cancers, such as those of the breast and prostate (Muir & Westcott, 2003; Goyal et al., 2014).

Conversely, flaxseeds also contain anti-nutrients, namely the cyanogenic glycosides linustatin, neolinustatin, and linamarin that may lead to adverse health effects. Cyanogenic glycosides are chemical compounds in foods that release hydrogen cyanide (HCN) when chewed or digested. They consist of nitrogen-substituted secondary plant metabolites that occur in a large number of plant species, including cassava and flaxseed (Vetter, 2000). If consumed, the cyanogenic glycosides can be hydrolyzed by intestinal *B*-glucosidase during digestion, leading to the release of toxic HCN (Muir & Westcott, 2003; Bacala & Barthet, 2007). The levels of cyanogenic glycosides in flaxseed (250 – 550 mg/100 g) (Singh et al, 2011) are typically too low to produce acute affects in humans, but concern regarding chronic health effects of flaxseed consumption places limits on the recommended level of flaxseed consumption (WHO, 2012; Strandas, 2008).

Several processing methods aimed at removing these undesirable compounds from flaxseed have been attempted. A solvent extraction method utilizing hexane was developed that substantially removes the cyanogens, but also reduces some of the desired nutrients, notably the lignans (Wanasundara et al., 1993). Furthermore, the utilization of hexane in food preparation is unacceptable to many consumers because of its negative effects on health and the environment (Gros et al., 2003). Two fermentation methods were successfully developed to remove cyanogenic glycosides from flaxseed meal. One relies upon steam evaporation and is, therefore, energy consumptive (Yamashita et al., 2007).

The other requires powdering the flaxseed meal and using enzymatic preparations containing human liver *B*-glucosidase and *Bacillus* species cyanide hydratase produced by a recombinant *Pichia* strain (Wu et al., 2012). This method lacks universal appeal because of its requirement for powdered flaxseed meal and its reliance on genetically modified enzyme strains. Another fermentation method using lactic acid bacteria (LAB) to degrade the cyanogenic glycosides in flaxseed meal had been attempted (Lei et al., 1999). The LAB fermentation method was able to degrade the cyanogenic glycosides in the fermented meal, but retained high levels of total cyanogens in the form of a more toxic intermediate. Therefore, a need remains to identify and evaluate other methods of degrading cyanogenic glycosides that may be may be more acceptable, cost and energy efficient, and potentially simpler.

A simple wetting method that was developed to reduce the cyanide content of cassava flour is now recognized by the World Bank, FAO, and WHO as an effective intervention technique for preventing diseases associated with dietary cyanide consumption (Banea, 2015; Bradbury, 2010). This technique has been used to reduce the exceptionally high cyanide levels in cassava flour by nearly 6-fold (Cumbana, 2007). However, the SWM has not been investigated for its

applicability to flaxseed. Therefore, this study was designed to determine if the wetting method significantly reduces the total cyanide content of ground flaxseed (flaxseed flour). The effect of the variables time, temperature, and exposed SA on % TCR was investigated with the objective of optimizing the SWM to reduce total cyanogen content in flaxseed flour. Additionally, the lignan secoisolariciresinol diglucoside (SDG), the compound that provides many of the health benefits attributed to flaxseed consumption, was quantified to assess whether the wetting method significantly affects the SDG lignan content of flaxseeds.

1. Literature review

1.1 Background

Flaxseed, also referred to as linseed, is an ancient crop with long history of cultivation and human consumption. It is believed to be among the first domesticated plants, cultivated in the fertile valleys of the Tigris and Euphrates rivers in Mesopotamia around 7000 BC (Oates, 1979; Zohary & Hopf, 1993). Historically, flaxseed had been consumed in cereal dishes, breads, and drinks and has been also valued for its medicinal qualities (see Table 1.1). The oil it provides is used variously as a culinary medium, industrially as a lamp oil, and as a preservative in flooring and paint products (Muir & Westcott, 2003; Goyal et al., 2014). Additionally, flax fibers were used in the manufacturing of linen fabric and strong sailcloth (Muir & Westcott, 2003). Hence, the Latin name *Linum usitatissimum,* which translates to "very useful," is well deserved.

Table 1-1. Medicinal uses of flax described in history

About 650 B.C.	Hippocrates, the father of medicine, advocated flax for the relief of abdominal pains; and Theophrastus recommended the use of flax mucilage as a cough remedy.
About 1st century A.D.	Tacitus praised the virtues of flax.
About 8th century A.D.	Charlemagne considered flax so important for health of his subjects that he passed laws and regulations requiring its consumption.
About 15h century A.D.	Hildegard von Bingen used flax meal in hot compresses for the treatment of both external and internal ailments.

(Source: Flax council of Canada (2012)
http://www.flaxcouncil.ca/english/index.jsp?p=what1&mp=what)

Today flaxseed is predominantly grown in Canada but also in China, India, the United States, and the European Union, with an annual production of 10.6 million tons (FAOSTAT, 2013). The health properties associated with flax have led to a resurgence of interest in increasing human flaxseed consumption, primarily as a functional food commodity. The major health benefits attributed to flaxseed consumption are reductions in the incidences of cardiovascular disease, diabetes, and hormone-sensitive cancers, but it has also been found to improve many other conditions (Goyal et al., 2014). These benefits are mainly attributed to the linoleic acids, lignans, plant proteins, and fiber mucilage provided by flaxseeds (Muir & Westcott, 2003).

The health promoting oil in flaxseed, α-linoleic acid, is an essential omega-3 fatty acid that represents about 57% of the total fatty acids in flax; it is believed to prevent arteriosclerosis, alleviate inflammatory conditions, and significantly decrease the risk for coronary heart disease (Dolecek, 1992; Djousse et al., 2001; Singh et al, 2011; Mayengbam et al, 2014). The lignans in flaxseed are found at extraordinarily high levels, at least 75 times greater than any other known plant source, with secoisolariciresinol diglucoside (SDG) being the predominant lignin found in flaxseed (Axelson et al., 1982; Thompson et al., 1991). The health benefits attributed to flaxseed lignans are a reduction in risk factors associated with hormone-dependent cancers such as breast cancer (Thompson et al, 2005; Muir & Westcott, 2003; Phipps et al., 1993) and prostate cancer (Denmark-Wahnefried et al., 2004). Lignan supplementation has also been shown to improve glycemic control in diabetic patients (Pan et al., 2007). The protein content in flaxseed meal is high-quality; it is comparable to that of soy, but in animal studies it was found to be more efficient at lowering total plasma cholesterol and triglycerides (Bhathena et al., 2002). Flaxseeds have similarly been found to lower total and LDL cholesterol levels (Katare et al., 2012). The soluble fiber content of flaxseed is also quite high, at 60-80 g/kg mucilage (Cunnane et al., 1993). Diets

high in fiber may help to reduce the incidence of heart disease, diabetes, colorectal cancer, obesity, and inflammation (Morris, 2003).

Numerous other scientific studies have supported these and other health benefits of flaxseed consumption (tabulated findings of a recent review article focused on evidence from human and animal studies evidencing a broader range of potential health benefits of flaxseed can be found in Goyal et al. (2007). Moreover, the diverse components of flaxseed seem to offer synergistic health benefits, and consumption of the whole flaxseed may provide additional benefits beyond that of its individual nutritional components (Goyal et al., 2014; Oomah, 2001; Styrczewska et al., 2013). Therefore flaxseed has established itself as a functional food, defined as a food or food ingredient that may provide physiological benefits beyond basic nutritional functions that help to prevent and/or cure disease (Kajla et al., 2015; Al-Okbi, 2005, Goyal et al., 2014). The recognition of the functional food status attributed to flaxseed was exemplified by the USDA funding research projects aimed at incorporating flaxseed into bread products; the stated goal being to incorporate this functional food into a common food item such as bread to help promote flaxseed consumption to reduce the risks of cardiovascular disease, cancer and diabetes (Conforti, 2003).

However, flaxseeds also contain anti-nutrients that adversely affect health and severely limit the recommended level of flaxseed consumption. Cyanogenic glycosides are the anti-nutrient of concern, present at levels of 250-550 mg/100 g in whole flaxseeds (Singh et al, 2011). Fortunately, these levels are considered too low to produce acute affects in humans, but there is longstanding concern regarding chronic health effects of flaxseed consumption (Muir & Westcott, 2003). If ingested, cyanogenic glycosides can be liberated as toxic hydrogen cyanide in the intestines via the activity of intestinal *B*-glucosidase (Kajla et al., 2015) which can result in toxicity toward the mammalian respiratory, nervous, and endocrine systems (Wu et al., 2008). Thiocyanates are

produced that interfere with iodine uptake by the thyroid gland and long-term exposure to these causes or aggravates iodine-deficiency disorders, primarily goiter and cretinism (Muir & Westcott, 2003; Kajla et al., 2015; Yamashita et al., 2006). Therefore, a provisional tolerable daily intake has been set at 12 µg cyanide/kg body weight (WHO, 1996), which limits the daily intake of flaxseed to 10-20 g whole flaxseed (Strandas, 2008) or about half that for flaxseed meal.

Consequently, several processing methods aimed at removing these undesirable compounds from flaxseed have been attempted. A solvent extraction method utilizing hexane has been proposed to remove the cyanogenic glycosides. However, this method neglects to completely remove the cyanogens while removing some of the nutrients, notably the lignans (Yamashita et al., 2005; Wanasundara et al., 1993). Furthermore, the utilization of hexane extraction methods in food preparation is unacceptable to many consumers because of its potential negative effects on health and the environment (Gros et al., 2003).

Other methods utilizing fermentation to remove cyanogenic glycosides from flaxseed meal have been developed. The most recent methods, one by Yamashita (2005) and the other by Wu (2012), successfully removed the total cyanogens from flaxseed, whereas a prior study by Lei (1999) did not. The older fermentation method by Lei (1999) attempted to remove cyanogenic glycosides from flaxseed using a "natural' fermentation method through the use of lactic acid bacteria (LAB) present in traditional fermentation systems, as these LAB have been shown to produce enzymes effective in detoxifying cyanogens (Lei et al., 1999; Ogbonnaya, 2015). Researchers successfully isolated LAB and yeast from cassava that could produce effective exogenous β-glucosidase (Lei et al., 1999). These microorganisms were able to degrade the cyanogenic glycosides in flaxseed, but not the total cyanogens because a toxic cyanohydrin intermediate breakdown product remained. The toxic intermediate could not be removed because cyanohydrins are stabilized by the characteristically low

pH achieved through LAB fermentation; cyanohydrin and HCN levels remained at nearly 50% of the original cyanogen level in the inoculated flaxseed experiment after fermentation time of 70 h (Lei et al, 1999). This study indicated that traditional LAB fermentation may not be the method of choice for removing total cyanogens from flaxseed.

The second fermentation study, by Yamashita et al. (2007) utilized a method involving an incubation period of 18 h at 30˚C allowing the enzymatic release of HCN and its ultimate removal by steam-evaporation. However, this method has the drawback of being energy consumptive because it requires the steam release of HCN at 120˚C for considerable time. On an industrial scale, this means that a steam-heated oven and steam room equipped with a recovery for steam vapor to absorb the generated HCN gas is needed (Yamashita et al., 2007). Furthermore, steam heating is not necessarily required, as altering the pH or increasing the SA could be used to volatize the HCN instead (personal communication with Bradbury 10/14/2015).

Another method recently developed by Wu et al. (2012) requires powdering the flaxseed meal and use of cofactors and an enzymatic preparation containing human liver β-glucosidase and a *Bacillus* spp. cyanide hydratase produced by a recombinant *Pichia* strain (Wu et al., 2012). These researchers developed an effective fermentation system optimized by response surface methodology (RSM). They were able to reduce the cyanide levels in flaxseed powder from 1.156 mg/g to 0.015mg/g after 48 h of fermentation under optimal conditions (25 g flaxseed powder, 50 mg cofactor $MgCl_2$, 50 mg cofactor $MnCl_2$, 1.27 g enzymatic preparation, 8.0 g water, pH 6.3 and 46.8˚C) (Wu et al., 2012). However, the low level of cyanogenic glycosides originally present in their flaxseed meal samples is of concern; this level is 2 to 5 times lower than the levels reported by other researchers (Muir and Westcott, 2003; Sigh et al., 2011) and the authors provided no remarks regarding the differences in cyanogenic glycoside levels or the ability of the method to perform well

with flaxseed containing the normal levels of cyanogenic glycosides. While this method is more energy efficient than the method of Yamashita (2007), it lacks universal acceptance because of its reliance on genetically modified enzyme strains. For example, food processed by this method may not be marketable in Europe (Regulation EC1829-2003).

Consequently, a need remains to identify and evaluate other biological methods of degrading cyanogenic glycosides in flaxseed that would be more acceptable, cost and energy efficient, and potentially simpler. For example, a SWM capable of reducing the total cyanide content in cassava flour (a food high in cyanogenic glycosides) up to 6-fold has recently been developed and recognized by the World Bank, FAO, and WHO as a sensitive intervention technique for preventing diseases associated with dietary cyanide consumption (Banea et al., 2015). The wetting method directly mixes cassava flour with water in nearly equal proportions and relies on endogenous β-glucosidase enzymes for cyanogenic glycoside conversion to HCN. It also requires spreading the mixture to a thin layer which maximizes the exposed SA and minimizes the depth, enabling HCN to readily volatize. Additionally, elevated temperatures have been shown to increase the rate of HCN removal from wetted cassava flour (Bradbury, 2006; Bradbury and Denton, 2010).

However, no published studies applying the SWM to flaxseed were found and utilization of this method for the removal of total cyanogens in flaxseed remains unevaluated. Therefore, this study was designed to determine if the SWM can significantly reduce the total cyanide content of ground flaxseed (flaxseed flour). The effect of the variables time, temperature, and exposed SA on % TCR was investigated with the objective of developing an optimal SWM for reducing total cyanogen content in flaxseed flour. Additionally, the content of the lignan secoisolariciresinol diglucoside (SDG), the compound that provides many of the health benefits attributed to flaxseed consumption,

was quantified to assess whether the SWM significantly affects the SDG lignan content of flaxseeds.

1.2 Cyanogenic Glycosides in Flaxseed

Cyanogenic glycosides are chemical compounds contained in foods that release hydrogen cyanide when chewed or digested. They consist of nitrogen-substituted secondary plant metabolites that occur in a large number of plant species, including flaxseed and other economically important crops. It is hypothesized that they evolved either as an important evolutionary defense mechanism (Bak et al., 2006; Naumann & Miller, 2004) or to provide a storage deposit of reduced nitrogen and sugar for the developing seedlings (Selmar et al., 1990). The chemical structure of cyanogenic glycosides is that of a reactive α-hydroxynitrile component stabilized through conjugation with either D-glucose or gentiobiose (6-O-β –D-glucopyranosyl-D-glucose) (B-glycosides of cyanohydrins) (Barceloux, 2009).

A Canadian study of oilseed types determined that there is a large variation in the levels of cyanogenic glycosides in flaxseeds, with most of the variation due to different cultivars as well as variation due to location and year (Ommah et al., 1992). Linustatin, neolinustatin, and linamarin are the cyanogenic glycosides found in flaxseeds (see Fig. 1-1). The primary flaxseed cyanogenic glycoside is linustatin, found to range from 213 to 352 mg/100 g of seed, followed by neolinustatin (91 to 203 mg/100 g seed); linamarin tends to be scantly present (< 32 mg/100g) (Ommah et al, 1992). These levels have been confirmed in other studies, in which the cyanogenic glycoside content in flaxseeds ranged from 250-550 mg/100 g (Sigh et al, 2011). However, cyanogenic glycosides increase under drought conditions – so global warming may result in an elevation in cyanogenic glycoside levels in cyanogenic plants (Brown et al., 2016).

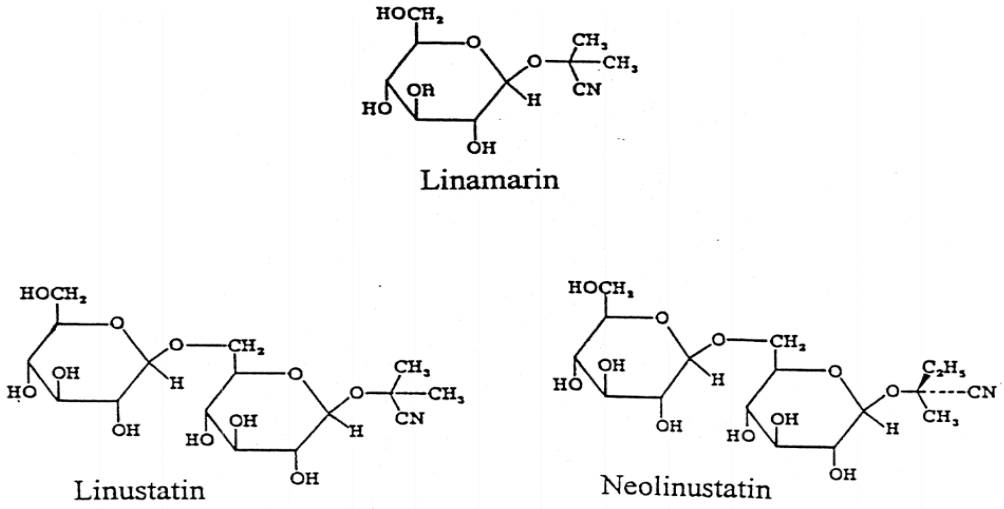
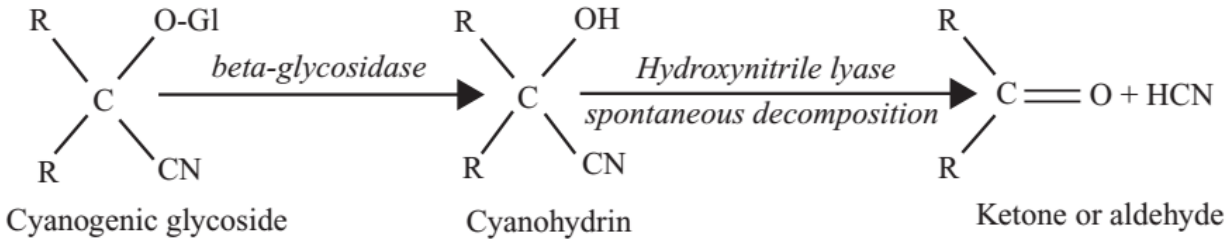


Figure 1-1. Molecular structures of the cyanogenic glycosides that occur in flaxseeds: linustatin, neolinustatin, and linamarin (Lei et al., 1999).

Plants that accumulate cyanogenic glycosides also produce the enzymes required to hydrolyze these glycosides (Vetter, 2000; Haque & Bradbury, 2002). However, in plant tissues, the intact cyanogenic glycosides are specifically compartmentalized so that they are separate from their hydrolyzing enzymes. In flaxseed, the cyanogenic glycosides are located in the cell vacuoles while the enzymes (β-glucosidases and hydroxynitrile lyases) are located in the mesophilic cells of the plant (Morant & Jorgensen, 2008). These endogenous β-glucosidases are released upon cell rupture due to maceration or grinding so that this hydrolyzing enzyme can then come into contact with the intact cyanogenic glycosides, resulting in the enzymatic cleavage of the carbohydrate D-glucose moiety. The α-hydroxynitrile is then liberated, which can then either be enzymatically cleaved by hydroxynitrile lyase or it can spontaneously dissociate, releasing HCN (Fig. 1-2) (Barceloux, 2009; WHO, 2012). Under the appropriate conditions, the HCN produced can volatilize, which poses no health threat if this is achieved prior to consumption.



Gl = glucose

Figure 1-2. General catabolic scheme for cyanogenic glycosides. (WHO, 2012)

The cyanogenic glycosides, their cyanohydrin breakdown product, and HCN are together referred to as total cyanogens. The cyanohydrins can remain in partially processed foods containing cyanogenic glycosides and their decomposition is pH dependent (WHO, 2012). It was concluded that the cyanohydrin behaves in vivo as its molar equivalent to cyanide (NAS, 2009). This is because the cyanohydrin intermediate has been shown to readily breakdown down to HCN in the mildly alkaline environment of the intestine (Mlingi et al., 1995). Thus, ineffectively fermented foods such as gari made from cassava (a food often high in cyanogenic glycosides) that retain high cyanohydrin levels pose a much greater threat to consumers than the original unfermented cassava because cyanogenic glycosides are less efficiently converted to HCN in vivo than are the cyanohydrin intermediates (Carlsson et al., 1999; WHO, 2012). Therefore, from a food safety perspective, the effectiveness of the cyanogenic glycosides conversion to the cyanohydrin intermediate and the subsequent conversion of this intermediate to HCN and its ability to volatilize are all particularly important steps to consider during food processing.

For effective detoxification of flaxseed, the pH of each step of cyanogenic glycoside catabolism (Fig. 1-2) is both important and diverse. Considering the three catabolic steps and their requirements: (i) the easier enzymatic hydrolysis of the cyanogenic glycoside to the cyanohydrin requires a nearly neutral pH, (ii) the breakdown of the cyanohydrin to release HCN is ideally

achieved under alkaline conditions (≥ pH 6 for spontaneous dissociation), which markedly slows at pH 5 and ceases at pH 4, and (iii) the volatilization of HCN will happen best in acidic conditions (WHO, 2012; Ganjewla et al., 2010) or through increasing the SA under higher pH conditions (Bradbury 2006). Furthermore, the cyanohydrin intermediate is stabilized by an acidic pH, such as that of the lactic fermentation where it tends to remain intact (Giraud et al, 1993; Lei et al., 199).

1.3 The Simple Wetting Method

1.3.1 Background

Cassava is a cyanogenic plant that is an important food source for many parts of the world. Its production is rapidly increasing, especially in sub-Saharan Africa, as it is a drought resistant plant that can grow in marginal soils. In years of low rainfall, the average ppm value of cyanide in cassava has been found to double (Ernesto et al., 2002) and this cyanide is retained as cyanogens for many years in cassava flour stored under ambient conditions (Bradbury, 2006). Consumption of high levels of cyanogens from cassava is associated with the development of Konzo, an irreversible paralysis of the legs that occurs mainly in children and young women that is increasing rapidly in certain parts of sub-Saharan Africa (Karumba et al., 2009; Banea et al., 2015). The association with dietary cyanide is evidenced by the % mean monthly incidence of Konzo having been significantly related to the monthly % of children with high urinary thiocyanate levels, which is a measure of cyanide intake (Banea et al., 2014). In 2005, Bradbury et al. developed a "simple wetting method" that reduced the total cyanide content of cassava flour up to 6-fold (Bradbury, 2006; Cumbana et al., 2007). The method can be used alone or in combination with other traditional methods for reducing the cyanide content of cassava. The SWM is now accepted by the World Bank, FAO and WHO as an established sensitive intervention for the reduction of Konzo incidence (Banea et al., 2015). Cassava produces two cyanogenic glucosides, linamarin and to a lesser extent lotaustralin, as well as the enzyme linamarase that catalyzes their breakdown to glucose and cyanohydrins. Under

certain conditions, the cyanohydrins can decompose spontaneously, liberating HCN and a ketone (Fig. 1-2) (Cumbana et al., 2007). The SWM described below outlines the conditions as well as techniques used to volatize the HCN and to minimize the retention of the toxic cyanohydrin intermediate.

1.3.2 Development of the Simple Wetting Method

Cassava flour samples were obtained from Mozambique, Indonesia, and the Plant Culture Facility of the Australian National University. The cassava flour was quite stable, and no change in its cyanide content was observable over a period of years when stored at -20°C. Experiments were done to determine the loss of cyanide from cassava flour under different time, temperature, and exposed SA conditions after several preliminary findings were obtained.

The preliminary studies tested the effects of ambient air conditions and relative humidity on the decrease in total cyanide content and these showed that there was no decrease in total cyanide content of flour samples left for 6 months under ambient laboratory conditions (Bradbury, 2006). However, the same flour samples in closed glass desiccators over excess water (100% RH) displayed a steady decrease in total cyanide over time (Cardoso et al., 2004). Other preliminary experiments investigated the effect of moisture content on the degradation of linamarin catalyzed by linamarase by using flour-to-water ratios of 1:1, 1:2, and 1:3. However, no observable difference in the degradation of linamarin was found among the different hydration levels tested, and subsequent studies employed a 1:1.25 flour-to-water ration. Since the presence of excess water did not increase the rate of linamarin degradation, it was concluded that once the flour reached its maximum swelling capacity, hydrolysis occurred at its maximum rate for that particular enzyme concentration (Bradbury, 2006). Preliminary experiments were also conducted to determine the effect of particle size on the degradation of linamarin. The flour was sieved into separate particles sizes of fine

(<425 µM), medium (425-850 µM), and coarse (>825 µM) flour samples prior to use and subsequent analysis after application of the wetting method showed that the range of particle sizes used had no effect on the outcome (Bradbury, 2006).

The standard experimental procedure then involved mixing cassava flour (5 g) with 6.25 mL water (pH ca 5.7) in a 100 ml beaker using a 1:1.25 proportion of flour to water (Bradbury, 2006). The water was absorbed by the flour and then duplicate 100 mg samples of wetted flour were removed at 0, 1, 2, 3, 5, and 24 hours from the open beaker for total cyanide analysis using the picrate method. The beaker was kept at 30˚C or 35˚C in a constant temperature oven. After each incubation period, the beaker was weighed and water was added back with mixing to replace that lost by evaporation.

Analysis for total cyanide of the cassava flour samples by use of the picrate method produced a quantifiable cyanide solution with absorbance measurable at 510 nm with a spectrophotometer (Bradbury, 2006). Total cyanide content was then calculated in ppm by multiplying the absorbance by 396 (Egan et al., 1998; Bradbury et al., 1999). The total cyanide content was converted to % of total cyanide remaining using the zero time value as 100% and plotted versus time of treatment by using the following equation:

% total cyanide remaining = (ppm cyanide at t = X) x 100/(ppm cyanide at t = 0). (Equation 1)

At a 5 h termination point, the experiments performed at 30˚C and 35˚C gave the same rate of breakdown, as depicted in Fig. 1-3 and Fig 1-4 below. Based on these data, 30˚C was used as the standard temperature for use in tropical countries. Using only the residual linamarase in the cassava flour, this standardized method yielded a 3-fold reduction in total cyanide content (Bradbury, 2006).

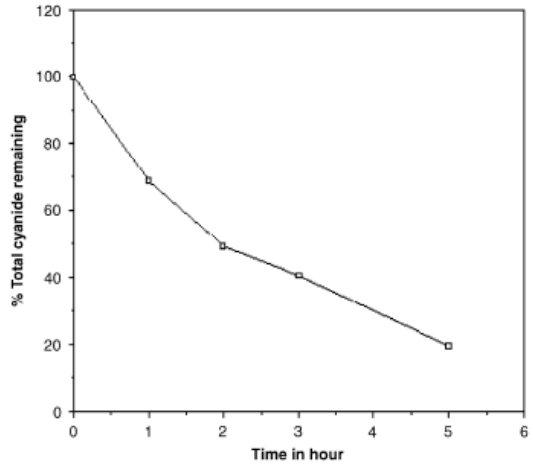


Figure 1-3. % total cyanide remaining versus time (h) for 5 g cassava flour sample, mixed thoroughly with water (1:1.25) and left in an open beaker at 30˚C. (Bradbury, 2006).

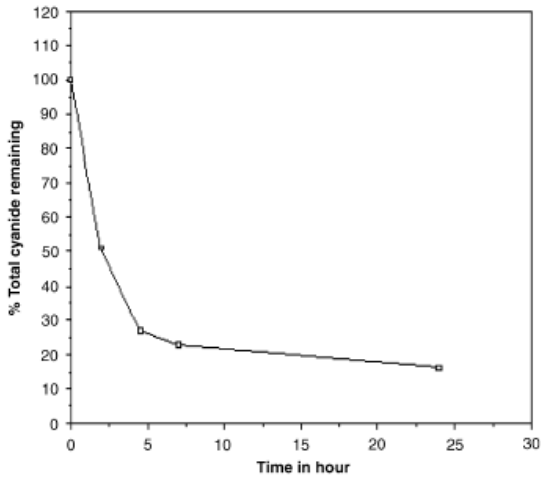


Figure 1-4. Graph of % total cyanide remaining versus time (h) for 100 g cassava flour from Mozambique mixed thoroughly with 125 mL water and left in an open beaker at 30˚C. (Bradbury, 2006).

Bradbury later performed other experiments to ascertain the combined effect of time, increased exposed SA, and elevated temperature on the % of total cyanide remaining in cassava flour (to approximate spreading the mixture into a thin layer and placing it in the sun) (Bradbury, 2006). The effect of increasing the exposed SA and minimizing depth to achieve a greater cyanide loss was investigated by spreading the damp flour to various thicknesses on a tray. It was found that 500 g samples spread into thin 0.5 cm depths had the lowest cyanide retention (18%) compared to thicker 1.5 cm depths (27%) and 4 cm depths (38%) (Bradbury & Denton 2010). Consequently, Bradbury (2006) concluded that it was important to increase the SA of the flour mixture by spreading it into

thin (0.5 cm) depths as this facilitated the removal of the water-soluble HCN gas (which may become trapped in the thicker depths resulting in less HCN volatilizing). Additionally, any accumulation of the weakly acidic HCN in the damp flour mass could also lower the pH and thus reduce the rate of breakdown of the cyanogenic glycoside linamarin (Cumbana et al., 2006). Furthermore, applying this method to cassava flour at 30˚C and 50˚C showed that temperature elevation resulted in greater total cyanide removal (Fig. 1.5). Bradbury hypothesized that this was likely due to the increased rate of breakdown of the acetone cyanohydrin to HCN at the higher temperature, referencing White et al. (1994) (Bradbury, 2006). When combined, a method utilizing a five h treatment time, 50˚C temperature, and increased SA (achieved by spreading the wetted mixture into a 0.5 cm depth) resulted in cassava flour with a 6-fold reduction in the total cyanide (Cumbana et al., 2006).

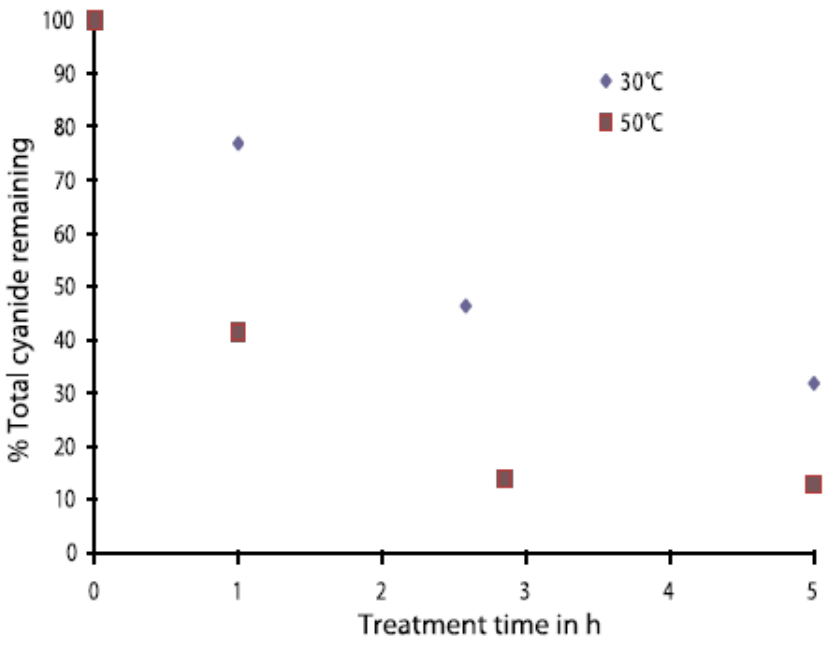


Figure 1-5. % Total cyanide remaining in wet cassava flour vs. treatment time (h) at different temperatures. (Bradbury and Denton, 2010).

1.4 Analytical methods used for the quantification of cyanide

1.4.1 Total cyanide potential

The three compounds of toxicological concern in foods produced from cyanogenic plants are (1) the cyanogenic glycosides, (2) the cyanohydrin intermediate breakdown product that can remain after processing and food preparation and (3) any non-volatized HCN/CN^- that remains in the food upon consumption. The cyanogenic glycosides are either quantified individually or the total HCN content of the cyanogenic glycosides and the cyanohydrins. The total HCN content is determined via a complete hydrolytic conversion of the cyanogenic glycosides to cyanohydrins, followed by quantification of the HCN subsequently released. Therefore, the "total HCN" term refers to the total HCN content of all cyanogenic glycosides, cyanohydrins, and liberated HCN present in a food (WHO, 2012).

1.4.2 Methods of measuring total HCN

The hydrolytic release of total HCN from cyanogenic glycosides in flaxseed, as in all cyanogenic plant foods, can be accomplished by spontaneous, endogenous hydrolysis in the macerated plant matter. This is possible because flaxseed contains two cyanogenic β-glucosidases, linustatinase and linamarase. HCN can also be liberated by adding the appropriate β-glucosidase enzymes to ensure that the cyanogenic glycosides are completely converted to cyanohydrins (Fig. 1-2). The cyanohydrins can subsequently decompose spontaneously (at appropriate pH or with increased SA) or through enzymatic assistance to HCN (Haque & Bradbury, 2002).

A drawback to relying on endogenous enzymes to release HCN is that some plant cultivars may either lack or be deficient in these enzymes. They may also be denatured through food processing. Adding supplemental enzymes to liberate cyanide may also have limitations, as specific

glucosidases are required for different cyanogenic glycosides. In the case of flaxseed (which contains linustatin, neolinustatin, and linamarin) two specific enzymes are required. Linustatinase first converts linustatin to linamarin and neolinustatin to lotaustralin. Then the second enzyme, linamarase, liberates cyanide from linamarin and lotaustralin (Bacala & Barthet, 2007). An alternative, non-enzymatic, method for determining total HCN is to use dilute acid with heating at 100°C to hydrolyze the cyanogenic glycosides to the corresponding cyanohydrins, which release HCN/CN^- upon basification. This method has the advantage of bypassing enzyme deficiencies and so is applicable to any plant material, as well as fresh and processed foods (Bradbury & Egan, 1994; Haque & Bradbury, 2002; ESR, 2010).

A picrate method has also been developed for determining the total cyanide content of flaxseeds. This method is based on the knowledge that flax, like most cyanogenic plants, contains the appropriate enzymes to ensure hydrolysis of the cyanogenic glucosides. The picrate method has been found to be as accurate and reproducible a method for measuring total cyanogens in flaxseeds as the acid hydrolysis method discussed above and was considered having the advantage of being less difficult to perform (Haque & Bradbury, 2002). However, the picrate method is dependent on the pH of the samples being near pH 6.

1.5 Toxicological impact of cyanogenic glycosides in animals including humans

1.5.1 Absorption, distribution, and excretion of cyanogenic glycosides

After oral consumption, a portion of the ingested cyanogenic glycosides will be absorbed and excreted intact in the urine. The unabsorbed fraction can be converted to HCN by β-glucosidase enzymes in the GI tract (Carlsson et al., 1999). In humans, thiocyanates are produced internally by the cyanogenic glycoside detoxification mechanisms (Fig. 1-6). Thiocyanates are used as biomarkers for cyanide exposure (WHO, 2012; Ganjewala et al., 2010).

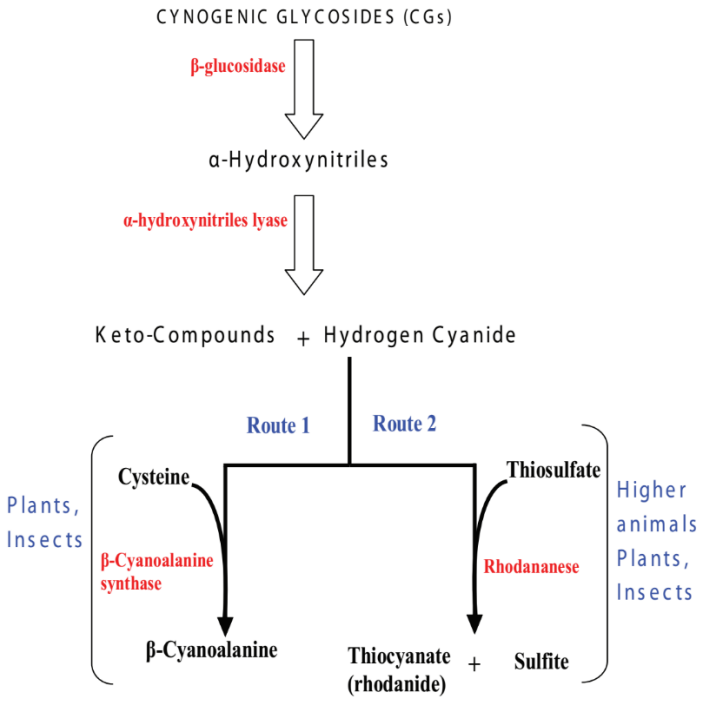


Figure 1-6. Schematic pathway of cyanogenic glycoside catabolism and production of thiocyanate in higher animals and plants (Ganjewala et al., 2010).

1.5.2. Observations and flaxseed dietary exposure assessment in humans

Some have claimed that cyanogenic glycosides can be removed through cooking or baking (Cunnane et al., 1993). The potential use of flaxseed as a human food is dependent on being able to maintain its beneficial attributes while decreasing potential toxicological concerns. An investigation of the human health benefits and food safety concerns related to flaxseed consumption showed that while consumption of muffins containing 50 g of flaxseed flour for 4 weeks lowered total serum cholesterol by 9% and low-density-lipoprotein-cholesterol by 18%, urinary thiocyanate excretion was raised 2.2-fold (Cunnane et al., 1993). Urinary excretion of thiocyanate was used as a biomarker of exposure to cyanogenic glycosides (WHO, 2012). Cunnane and co-workers were unable to detect cyanogenic glycosides (total cyanogens were not measured) in the flaxseed flour muffins, leading them to hypothesis that the cyanogenic glycosides were destroyed during baking (Cunnane et al., 1993). However, the increase in thiocyanate excretion suggests that the total

cyanogens were not destroyed and/or detected, i.e. biologically active cyanohydrin or HCN may have remained after baking.

A recent evaluation of the cyanogenic glycosides in plant-based foods available in New Zealand found that flaxseed contained the highest concentration of hydrocyanic acid (91-178 mg/kg (Cressey et al., 2013). Flaxseed-containing breads were found to contain total cyanogens at concentrations expected from their flaxseed content, levels of total cyanogens were consistently high (> 50 mg/kg) and were similar to previously published materials on cyanogenic content level of flaxseed. These results were interpreted as indicating that the baking process has little or no impact on the total cyanogenic glycoside content of flaxseed-containing breads. And while the total cyanogenic content of flaxseed-containing breads in New Zealand is modest, it was deemed to be of potential concern because bread is a product that is consumed frequently and in significant quantities (Cressey et al., 2013). This concern was shared with the Joint FAO-WHO Expert Committee Report on Food Additives (JECFA) (WHO, 2012). That committee concluded that more extensive occurrence data related to cyanogenic glycosides is needed. They stated that data needs would ideally include data showing the ratio of cyanogenic glycosides to cyanohydrin to HCN in raw and processed versions of a wide range of foods containing cyanogenic glycosides (WHO, 2012).

2. Research questions, goals and hypotheses

2.1. Research questions to be addressed.

While the WHO has approved the SWM developed by Bradbury and his co-workers to significantly degrade the cyanogenic glycosides in cassava, there is a lack of information on whether this simple technology could be used to reduce the associated elevated levels of cyanogenic glycosides in flaxseed. Such information is needed if we will be able to take advantage of this potential source of

food, particularly in developing countries. Accordingly, the current research is designed to address the following questions:

a. What modifications of the SWM developed by Bradbury and colleagues for cassava are needed to make the techniques equally suitable for flaxseed flour?
b. What effect does lengthening the processing time have on the total cyanide reduction achieved by the SWM of flaxseed?
c. What is the effect of altering the temperature on the total cyanide reduction achieved by the SWM when applied to flaxseed?
d. What effects does SA have on the total cyanide reduction achieved by the SWM when applied to flaxseed?

2.2 Hypothesis and Objectives:

Two broad hypotheses are being addressed in the current study:

2.2.1. The SWM process currently being used for the degradation of cyanogenic glycosides in cassava will, with minimal modification, be effective in reducing the cyanogenic glycosides in flaxseed flour.

2.2.2. Optimization of the application of SWM to flaxseeds will require consideration of the effects and interactions of processing time, processing temperature, and the exposed SA of the wetted flaxseed mixture.

The hypotheses will be evaluated by addressing the five research objectives below:

Objective 1: Determine the effect of processing time on the effectiveness of the SWM as applied to flaxseed flour.

Objective 2: Determine the effect of processing temperature on the effectiveness of the SWM as applied to flaxseed flour.

Objective 3: Determine the effect of the exposed SA of the wetted flaxseed mixture on the effectiveness of the SWM as applied to flaxseed flour.

Objective 4: Determine potential synergies between the effects of processing time, processing temperature and the exposed SA of the wetted flaxseed mixture on maximizing the effectiveness of SWM as applied to flaxseed flour.

Objective 5: Determine the effect of SWM treatment of flaxseed on the retention of SDG lignan content.

3 Methods

3.1 Experimental Design

The total cyanogen content (TCC), cyanogenic glycosides, cyanohydrins, and HCN of whole ground flaxseeds (flaxseed flour) were quantified using the picrate method (Haque & Bradbury, 2002) before and after applying the SWM to measure cyanide reduction. Since this method relies on β-glucosidase enzymes (linustatinase and linamarase) to hydrolyze the cyanogenic glycosides present in flaxseed, an assay was also incorporated to measure endogenous flaxseed β-glucosidase activity. Finally, lignan secoisolariciresinol diglucoside *(*SDG) content of the flaxseed was measured before and after SWM treatment to assess the impact of the treatment on the retention of this desired compound.

This study includes experiments designed to determine the effects and interactions of (a) time, (b) temperature, and (c) SA of the wetted flaxseed mixture on the reduction of cyanogenic glycosides.

All experiments were repeated on three separate days as a means of assessing between experiment error, and final examination of flaxseed samples were performed in triplicate to assess the within experiment error. General linear mixed models (GLMMs) were used for statistical analysis using R. Differences were considered significant if $P<0.05$.

3.2. Collection and Preparation of Flaxseed Samples

Flaxseed was purchased in bulk from a local health food store (My Organic Market, 9827 Rhode Island Ave, College Park, MD 20740, phone (301) 220-1100) and was stored in a -20°C freezer at the BHNRC USDA. This seed was used to prepare freshly ground flaxseed flour before each analysis as described below.

3.2.1. Method for Preparing Flaxseed Flour

1. Seeds were thoroughly mixed to provide homogenous samples.
2. The flaxseed flour was prepared by finely grinding flaxseeds using a coffee grinder (Kitchen Aid, Model A-9 Coffee Mill) (Haque & Bradbury, 2002). Fresh flaxseed flour was prepared just prior to each experimental trial.

3.3. Measurement of β-Glucosidase Activity in Endogenous Flaxseed Enzymes

Beta-*g*lucosidase is an enzyme that catalyzes the hydrolysis of the terminal, non-reducing B1→4 bond linking two glucose molecules (such as p-nitrophenyl-β-D-glucopyranoside or PNPG). In this assay, PNPG is hydrolyzed specifically by β-glucosidase into a yellow colored product (β-D-glucose). The rate of appearance of this product is directly proportional to the β-glucosidase activity of the flaxseed sample and has a maximum absorbance at 400 nm.

p-Nitrophenyl-β-D-glucoside (PNPG) $\xrightarrow{\text{β-glucosidase}}$ *p*-Nitrophenol (PNP) + D-Glucose

The appearance of *p*-nitrophenol was measured spectrophotometrically at 400 nm. The β-glucosidase activity of the flaxseed flour was measured through the aid of a β-gluocosidase activity assay kit (Sigma Aldrich, MAK 129) as described below.

3.3.1. Assay for measuring β-glucosidase activity in endogenous flaxseed enzymes

3.3.1.1. Sample Preparation

The enzyme activity assay was a modification of the method of Esen & Blanchard (2000). A 50 mM phosphate extraction buffer (pH 7.0) was prepared by dissolving 0.29 g of monosodium phosphate and 0.77 g of disodium phosphate in 100 ml of distilled water. The flaxseed was ground with a coffee grinder (Kitchen Aid, Model A-9 Coffee Mill). One gram ground flax seed samples were mixed with 3.0 ml of extraction buffer in 15-ml centrifuge tubes, which were then held on ice for 30-60 min, swirling at 10 min intervals to suspend the settled matter. The samples were then centrifuged at 17,000g for 15 min. The supernatant was then transferred to a fresh centrifuge tube and the pellet was re-suspended in 1.5 ml extraction buffer. The re-suspended pellet was centrifuge again at 17,000g for 15 min. The two supernatants were combined and used for the enzyme activity assay. The unused portion of supernatant was frozen (-20˚C).

3.3.1.2. Assay Reaction

Distilled water in 20 µl portions was added to the wells in 96-well plate. Controls consisted of 200 µL distilled water added to one well and 200 µL of "Calibrator" to a second. The "master reaction mix" was prepared accordingly manufacturer's instructions (Table 3-1). The volume shown is enough for one assay well and has a final concentration of 1 mM β-NPG. The master reaction mix was prepared fresh each time the assay was run.

Table 3-1. Composition of the "master reaction mix."

Reagent	Volume
Assay Buffer	200 µL
B-NPG Substrate	8 µL

The assay was run by transferring 20 µl and 200 µl of the master reaction mix to a sample well. The plate was then tapped briefly to mix the components. The initial absorbance at 405 nm $(A_{405})_{initial}$ was measured immediately and recorded. The samples were incubated for 20 min at either room temperature or 37˚C for 20 minutes, the final absorbance measurement $(A_{405})_{final}$ taken, The enzyme activity was calculated using the equation:

$$\text{B-Glucosidase Activity (Units/L)} = \frac{(A_{405})_{final} - (A_{405})_{initial} \times 250 \text{ units/L}}{(A_{405})_{calibrator} - (A_{405})_{water}}$$

Where:

$(A_{405})_{calibrator}$ = value for calibrator at 20 minutes

$(A_{405})_{water}$ = value for water at 20 minutes

If the $(A_{405})_{final}$ was higher than 1.0, the sample was diluted with water and the assay repeated. One unit of β-glucosidase is the amount of enzyme that catalyzes the hydrolysis of 1.0 µmole of substrate per minute at pH 7.0.

3.4. Application of the SWM to Flaxseed Flour

The SWM was applied to flaxseed flour at room temperature (≈ 22˚C) for 24 h. To initiate the wetting method, the flaxseed flour was mixed with water at a hydration level of 1 part flaxseed flour to 2 parts water (Bradbury, 2006). The %TCR in the samples was determined by the picrate method (see 3.5). The total cyanide content in ppm is calculated by using the Equation 2

Total cyanogen content (ppm) = 396 X absorbance (Equation 2).

A plot was made of % total cyanide remaining versus time (h) through the use of Equation 1, where t = time (h) and X = duration of treatment (h) at 22˚C.

$$\% \text{ total cyanide remaining} = \frac{\text{(ppm cyanide at t = X) x 100}}{\text{(ppm cyanide t = 0).}} \quad \text{(Equation 1).}$$

This analysis was repeated on three separate days.

3.4.1. Basic Method for Measuring Cyanide Loss by Wetting Method

5g of ground flaxseed was mixed with 10 mL DI water in a 100 mL beaker until the water was absorbed (≈ 1 m). The beaker was placed under a hood kept at 22˚C for 24 hours, and was sampled at specified time points for cyanide analysis. Evaporative water loss was adjusted for at each sampling time point by weighing the beaker plus the flax solution at time 0 (before removing samples) and at each sampling time point (before removing samples). Water was added with mixing to replace any lost by evaporation and the weight of the removed samples was accounted for. Triplicate 100 mg samples of wet flaxseed flour were removed at 0, 1, 2, 3, 4, 6, 8, 10, 12, and 24 h (or other selected time points) from the open beaker. Samples were analyzed for total cyanide by the Picrate Method (see 3.5). A plot of % TCR was made by measuring the total cyanide in the flaxseed flour at the beginning of the wetting method experiment (t = 0) and at the termination time (t = X), and calculating the % TCR through the use of Equations 1 and 2. This analysis was repeated on 3 separate days.

3.5. The Picrate Method for Measuring Total Cyanide Remaining in Flaxseed Flour

Initial picrate kits for flaxseed were purchased from Dr. Bradbury at Australian National University. These kits were purchased in order to obtain the required linamarase control, which is no longer supplied by laboratory chemical manufacturers. The linamarase was then used as a control to validate the method in self-made kits, which were made following an outlined procedure (Haque & Bradbury, 2002). Methods for determining total cyanide remaining in flaxseed, preparing an appropriate buffer, and preparing the picrate measuring strips were adapted from the those outlined by Bradbury and co-workers (Bradbury & Bradbury, 1999; Egan et al., 1998).

3.5.1. Method for Determination of TCR in Flaxseed Flour.

Flaxseeds contain the cyanogenic glycosides linustatin, neolinustatin, and linamarin as well as the endogenous enzyme linustatinase that catalyzes the hydrolysis of these compounds, liberating HCN. The cyanogenic glycosides and hydrolytic enzymes are differentially compartmentalized in the flaxseed, so the flaxseed must be ground into flour before analyzing (see 3.2). To determine the TCR in the ground flaxseed, the following series of steps were performed:

1. 100 mg of ground wetted flaxseed flour were weighed out into a flat bottomed plastic bottle (25 mm diameter, 50 mm high).
2. 0.5 mL of 0.1 M phosphate buffer (pH 6) was added.
3. A 3 cm x 1 cm picrate paper (previously prepared and attached to a plastic strip) was placed in the bottle with the plastic oriented toward the base; care was taken to not allow the picrate paper to touch the liquid in the bottle.
4. The bottle was immediately closed with a screw cap lid.
5. For each set of experiments, another bottle was prepared (as above) without the ground flax seed, to serve as a blank.
6. A control was also run for each set of experiments. This was prepared placing a linamarin disk (containing 50 ppm linamarin and purchased with the kit), 0.5 ml of 0.1M phosphate buffer (pH 6), and a picrate paper adjoined to plastic strip (placing it as described above) into a bottle. The bottle was immediately closed with a screw cap lid.
7. The bottles were allowed to stand for 16-24 hours at room temperature (20-30˚C).
8. The plastic backing sheet from the picrate paper was then removed and the picrate paper from the sample was immersed in 5.0 mL of water for approximately 30 min with occasional gentle shaking.
9. Using a spectrophotometer, the absorbance at 510 nm of the picrate solution of the sample against that of the blank was measured.
11. The control was measured and confirmed to give the expected value (50 ppm linamarin).

12. The total cyanogen content in ppm was then calculated through the use of equation 2 and the %TCR was calculated through the use of equation 1.

3.5.2. Method for Preparing Buffer

A 0.1 M phosphate buffer (pH 6) was used. The buffer was prepared by mixing stock solutions of sodium phosphate dibasic (Na_2HPO_4) and sodium phosphate monobasic (NaH_2PO_4) prepared as follows:

A stock solution of solution A and solution B for sodium phosphate buffer was prepared. Solution A was prepared by dissolving 27 g of NaH_2PO_4 in a 1000 ml volumetric flask and filling to the 1000 ml line. Solution B was made by dissolving 53.65 g of Na_2HPO_4 in 1000 ml volumetric flask and filling to the 1000 ml line. The 0.1 M phosphate buffer (pH 6) was then prepared by referring to the chart below:

	NaH_2PO_4	Na_2HPO_4
Desired pH	Solution A (ml)	Solution B (ml)
6.0	87.7	12.3

Solutions A and B were mixed according to the chart and, were adjusted to pH 6 through the use of a pH meter.

3.5.3. Method for Preparing Picrate Papers.

Picrate papers were made by weighing 1.5 g moist picric acid (BDH Lab Reagent) and dissolving it in 100 ml of 2.5% (w/v) sodium carbonate. The 2.5% (w/v) sodium carbonate was made by dissolving 2.5 g of sodium carbonate in 100 ml of water. A square (10 cm x 10 cm) of Whatman 3MM filter paper was immersed in the picrate solution for 20 s and air dried. Once dried, the paper was cut into 3 x 1 cm rectangular pieces which were subsequently glued using a polyvinyl acetate adhesive to a plastic strip (10 mm x 50 mm). These were stored in a freezer.

3.6. Effect of Time and Temperature on % Cyanide Remaining in Flaxseed Flour.

Temperature influences the rate of breakdown of the cyanogenic glycosides (linamarin, linustatin, and neolinustatin) by the endogenous β-glucosidase and hydroxynitrile lyase enzymes in flaxseed. Furthermore, the spontaneous breakdown of the acetone cyanohydrin in flaxseed has been shown to occur at temperatures above 30°C (Vetter, 1999). Therefore, the effect of temperature variation on the %TCR in the flaxseed flour during application of the SWM was determined by carry out experiments at 55°, 65°, and 75°C with measurements taken at specified time points.

The mid-range temperatures were selected because linamarase activity has been shown to be optimal in the 55-60°C range (Mkpong et al., 1990). The maximum temperature was selected because excess heat was expected to denature the required enzymes, so that little cyanogenic glycoside breakdown was expected to occur at a temperature near 80°C (Bradbury & Denton, 2010) and the wetting method will, therefore, be ineffective.

The duration of the experiments will initially be for 5 hours at each temperature level, with measurements taken hourly to determine the decrease in the %TCR in the flaxseed flour sample (see Bradbury & Denton, 2010). This data will be used to select the optimal time and temperature for the wetting method to be used in the subsequent experimental section on effects of SA on total cyanogen reduction.

The total cyanogen content (ppm) and the %TCR in the flaxseed flour were calculated as described (see 3.5). This analysis was repeated for each temperature on three separate days. This data was then used to select the optimal time and temperature for the wetting method to be used in the subsequent experimental section on effects of SA on TCR.

3.6.1 Method for Determining the Effects of Time and Temperature on the Ability of the SWM to Decrease TCR in Flaxseeds Flour

The following series of steps were performed:

1. A laboratory oven (VWR Gravity Convection Oven, Model 414005-106) was set at the appropriate temperature (55°, 65°, or 75°C) and allowed to reach the set temperature.
2. 5g of flaxseed flour was mixed with 10 mL DI water in a 100 mL beaker until the water was absorbed by the flax (≈ 1 minute). The weight of beaker plus flax solution at time 0 (before removing samples) was recorded.
3. Triplicate 100 mg samples of wet flaxseed flour were removed at time 0 and prepared for later TCR analysis using the Picrate method. The weight of beaker plus flax solution was recorded at time 0 (after removing samples).
4. The open beaker was placed in the oven. The beaker was removed and weighed at the next hour time. Evaporative water loss was adjusted for by adding water back to the beaker with mixing.
5. Triplicate 100 mg samples of wet flaxseed flour were removed and prepared for later TCR analysis using the Picrate method. The total remaining sample plus beaker weight was recorded.
6. The methodology was repeated from step 4 for each hour time point, continuing for 5 hours (or other selected duration). Samples were then analyzed for TCR using the Picrate method and equations 1 and 2 (see 3.5).

   The hydration level was monitored while samples were in the oven so that the samples did not dry- out. The optimal time and temperature for processing flaxseed flour was determined by selecting the time and temperature that corresponded to the lowest %TCR. This optimal time and temperature was selected for the subsequent experimental section that evaluates the effects of SA on TCR in flaxseed flour.

3.7 Effects of Surface Area on % Cyanide Retention in Flaxseed Flour

Three different SAs were challenged for their effect on cyanide reduction in flaxseed at both 22°C and the selected optimal temperature (see 3.6). The SAs were considered to be the exposed surfaces generated through spreading 750 g of the wetted flaxseed mixture (250 g flaxseed flour and 500 g water) out either onto two large trays to a thickness of 0.5 cm, onto a smaller tray to a thickness of 2 cm, or placing the mixture into a laboratory beaker with a resulting thickness of 10 cm. For convenience, the different SAs were referred to by their thicknesses as thin (0.5 cm), thick, (2 cm) and cylinder (10 cm). However, the actual areas of the exposed surfaces for each tray or beaker were 2000 cm^2 (total volume 1000 cm^3 when spread 0.5 cm in thickness) for the large tray referred to as "thin," 415 cm^2 (total volume 830 cm^3 when spread to a thickness of 2 cm) for the smaller tray referred to as "thick," and 80 cm^2 (total volume 785 cm^3 for a 10 cm depth) for the beaker referred to as "cylinder."

This analysis was repeated on 3 separate days. The effect of SA on the efficiency of the SWM determined by statistically comparing the %TCR in the thin (0.5 cm), thick (2.0 cm), or 10 cm depths at each temperature over time (see 3.10). This data was used to select the optimal SA for decreasing the TCR in flaxseed flour.

3.7.1 Method to Measure the Effects of Surface Area on % Total Cyanide Retention in Flaxseed Flour.

The time and temperature were adjusted according to the experimental findings (see 3.6). A 250 g sample of ground flaxseed flour was mixed with 500 mL water in a large cylindrical beaker. Another two 500 g samples of flaxseed flour were mixed with 1000 mL water and spread out on a tray to a thin depth of 0.5 cm or to a thick depth of 2.0 cm. For each SA treatment, triplicate samples were removed and analyzed for TCR at time zero and at other h intervals over the time course (see 3.6) of the experiment and also at extended treatment times using the Picrate method (see 3.5). This was done at both 22°C and at the

selected optimal temperature for reduction in TCR (see 3.6). Procedures for determining %TCR were followed (see 3.5) using the Picrate method and equations 1 and 2. For both 22°C and the selected optimal temperature, the SA depth with the lowest measured %TCR was determined and selected as the optimal SA. This methodology was adapted from (Bradbury & Denton, 2010; Cumbana et al., 2007).

3.8 Measurement of Lignan Secoisolariciresinol Diglucoside (SDG) Content in Flaxseed Flour

The lignan secoisolariciresinol diglucoside (SDG) content was measured in the original flaxseed flour and this was compared to the lignan content of the flaxseed flour processed by the SWM at 22°C and at the selected optimal temperature (see 3.6). High-Performance Liquid Chromatography (HPLC) was used to determine the SDG concentration in the pre- and post-processed samples in order to confirm that the SWM, used with the intent to significantly reduce total cyanogen content in flaxseed, did not also significantly reduce the desirable SDG lignan content in the flaxseed flour. The samples were run in duplicate and compared for percent change. The HPLC method (adapted from Johnsson et al. (2000) involved steps of extraction, base hydrolysis, solid-phase extraction and HPLC quantification as depicted in Figure 3-1 and detailed in methodology steps 1-4. The method was developed for quantifying the SDG content of Soxhlet defatted flaxseed, but it was successfully applied to whole ground flaxseeds.

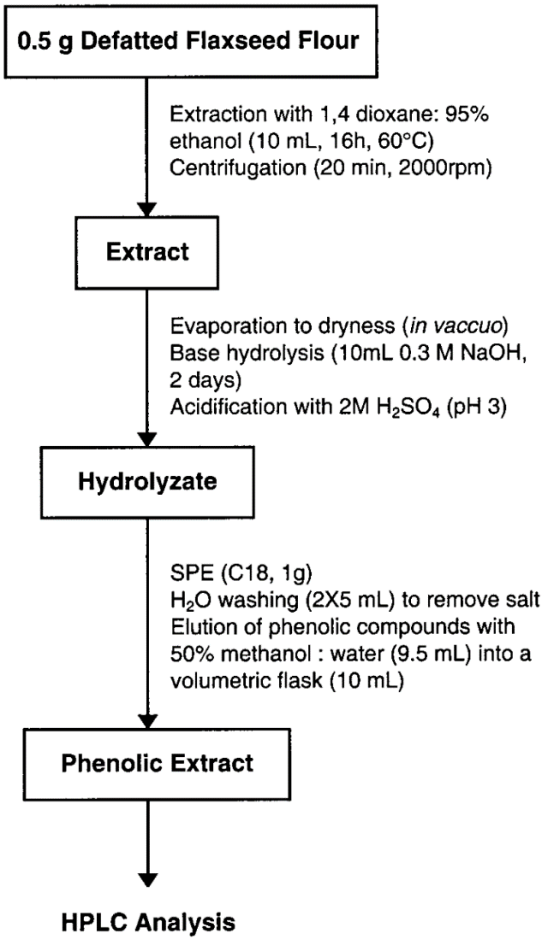


Figure 3-1: Schematic summary of the analytical methodology for analyzing SDG in flaxseeds (Johnsson et al., 2000).

3.8.1 Method for High-Performance Liquid Chromatography Analysis of SDG in Flaxseed.

Flaxseed samples were ground (see 3.2) and prepared for HPLC analysis as detailed in steps 1-3.

Step 1. A standard curve was generated.

A reference standard of SDG (Sigma-Aldrich) was purchased and a linear HPLC standard calibration curve was generated from HPLC analysis of 12 dilutions of stock SDG ranging from 0 to 100 µg. The standard calibration curve was used to generate an equation ($y = 4410.2 x - 164.46$, $R^2 = 0.9999$) used to convert peak areas (PA) to concentrations when solved for x and multiplied by PA.

Step 2. Extraction of SDG from the Defatted Flaxseed Flour

A 3 g sample of the wetted flaxseed flour was measured and SDG was extracted with 20 mL 1, 4-dioxane/95%ethanol (1:1, v/v) in test tubes by shaking for 16 hrs in a 60˚C water bath. The tubes were centrifuged (200 rpm, 20 min), washed, re-centrifuged, and the liquid phase was evaporated to dryness at 40˚C. The extracts were then subjected to alkaline hydrolysis for 2 days under constant rotation using 0.3 M aqueous sodium hydroxide. Subsequently, samples were acidified to pH 3 using 2 M sulfuric acid and the volumes were adjusted to 25 mL in a volumetric flask.

Step 3. Solid Phase Extraction (SPE) of an SDG-Rich Fraction

The salt was removed from the samples before HPLC analysis using a C18 reversed-SPE column (Mega Bond Elut, 6 mL/g, Varian Harbor City, CA) conditioned with 5 mL of methanol followed by 5 mL of water. 5 mL volumes of the hydrolyzed samples were then passed through the conditioned columns and the columns were washed with water (2 x 5mL) to remove the salt. The SDG-rich fraction was eluted with 9.5 mL of 50% aqueous methanol and the volume was adjusted to 10 mL in a volumetric flask. The recovery of SDG from the SPE column was then determined for each sample by HPLC analysis.

Step 4. High-Performance Liquid Chromatography Analysis

HPLC analysis was performed using 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. An Econosil RP C18, 5 *u*m, 250 x 4.6 nm (Alltech, Deerfield Il) was used with the column thermostat set to 25˚C and injection volume set to 10 *u*L. 5% acetonitrile in 0.01 M phosphate buffer, pH 2.8 (solvent A) and acetonitrile (solvent B) mixed A/B (v/v) were used for the mobile phase: 0 min (100:0), 30 min (70:30), and 32 min (30:70)

at a flow rate of 1 mL/min. Peaks were identified and SDG quantified by comparing them to those of the linear HPLC calibration curve. The standard curve equation was solved for X and this was used to convert the peak values to concentration of SDG.

Step 5. Compare the Original SDG Content to the Processed SDG Content

The original SDG lignan concentration of the ground flaxseed flour was compared to the SDG lignan concentration of the ground flaxseed flour processed by the optimal wetting method. The present retention was then determined.

3.9. Growth of Bacteria during SWM Processing

Mesophilic aerobic plate counts of the flaxseed was conducted for untreated samples and for samples subjected to the SWM at 22˚C for the selected optimal treatment time. The samples were plated on tryptic soy agar and incubated at 37˚C for 24 h. Total colony forming units (CFYs) from unprocessed samples and processed samples were then compared.

Wetted flaxseed samples were collected before processing. Other samples were collected from flaxseed processed by the SWM at 22˚C at end of the determined optimal treatment time. Samples were diluted 1:9 in duplicate (i.e., weigh out 3 gram of the wetted flax mixture and add 7 grams DI water). A serial dilution was made for both unprocessed (UP) and processed samples (PS) in duplicate (the dilution may need to be increased if growth exceeds ability to clearly count the CFUs). This was done by taking 1 ml of the above and adding it to 9 ml DI water sequentially.

50 µl samples were plated onto 16 tryptic soy agar plates with the aid of a spiral plater as follows:

UP Sample (1)	PS Sample (2)
Flax 1 10^o	Flax 2 10^o
Flax 1 10^1	Flax 2 10^1
Flax 1 10^2	Flax 2 10^2
Flax 1 10^3	Flax 2 10^3

The plates were incubated at 37˚C for 24 h and were enumerate with the aid of a plate reader. The CFU/ml were calculated [CFU/ml = (# of colonies x df)/volume used].

3.10 Procedure for pH Determination of Wetted Flax Material

The pH of the wetted flax material was measured on unprocessed samples and on samples collected at the end of the experimental procedures according to AACC Method 02-51. To determine the pH of the samples, 3 g of wetted flax material (composed of 1 g ground flaxseed and 2 g DI water) were placed into a 50 ml beaker and 8 ml of DI water (25˚C) was added. The wetted mixture was agitated until an even suspension was formed. The mixture was allowed to sit for 30 m, with intermittent agitation (to keep the particles in suspension). The mixture was then left to settle for 10 m, afterward the pH of the supernatant was determined with the aid of a pH meter.

3.11 Statistical Analysis

To determine the significance of the SA thicknesses over time on %TCR at each temperature, data were analyzed statistically using general linear mixed models (LMMs). For each temperature, %TCR was modeled as the response variable, with treatment time and specific SA thicknesses included in the model as fixed effects and experimental replicates included as random effects. The model was fit to a Gaussian family distribution using maximum likelihood, and the satisfaction of model assumptions were verified graphically. To determine the significance of each fixed effect, the full model was compared to a model in which that factor was dropped using a likelihood ratio test. The factor was retained in the final model when its inclusion in the model yielded a significantly better fit to the data. Factors were re-evaluated (i.e., with a different factor depth set as the baseline) and the model re-run to obtain pairwise comparisons between all factor depths. P values were calculated based on degrees of freedom calculated using the Kenward-Roger approximation (Halekoh & Højsgaard (2014). Differences were considered significant if $P<0.05$. Statistical

analyses were conducted using the lme4 packages (Bates et al. 2015) in R (R Core Team 2016). (See Appendix 1 for code).

4. Results

4.1. Cyanide Reduction

Initial experiments conducted at ambient temperature (~22˚C) on 5-gram flaxseed samples showed a strong correlation ($r^2 > 0.987$) between replicates performed on different days and were able to achieve an average TCR reduction of 96 % TCR when treatment time was 24 h and a minimum removal 89.4 % TCR at 20 h (Fig. 4-1). Kinetically, an initial rapid decline in TCR was observed up to 6 h, after which the rate of reduction declined from 6 h to 24 h.

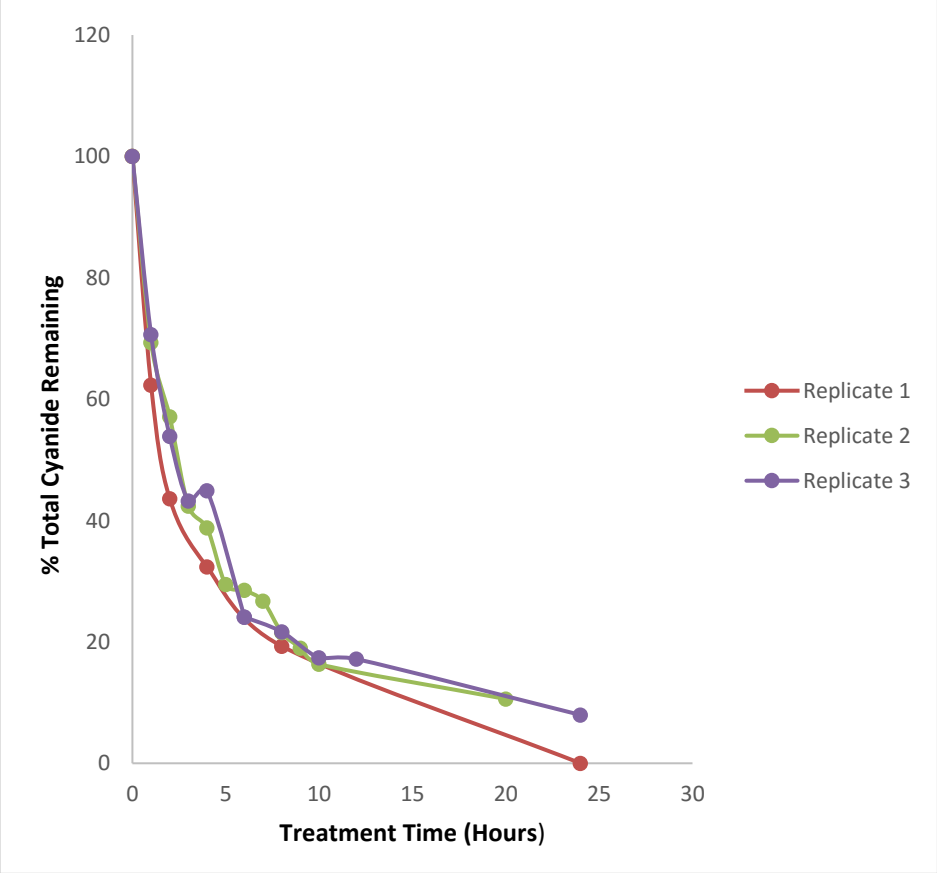


Figure 4-1. % Total cyanide remaining (TCR) in 5-grams wetted flaxseed flour vs. time of treatment at 22˚C. The three trials were performed in triplicate on separate days to determine the between experimental error.

The effect of performing the SWM protocol at elevated temperatures (55˚, 65˚, and 75˚C) in a similar manner with 5-gram samples is depicted in fig. 4-2. Correlation values between experimental trials showed close agreement, with correlation coefficients (r^2) ranging from 0.91 to 0.99 (average = 0.97).

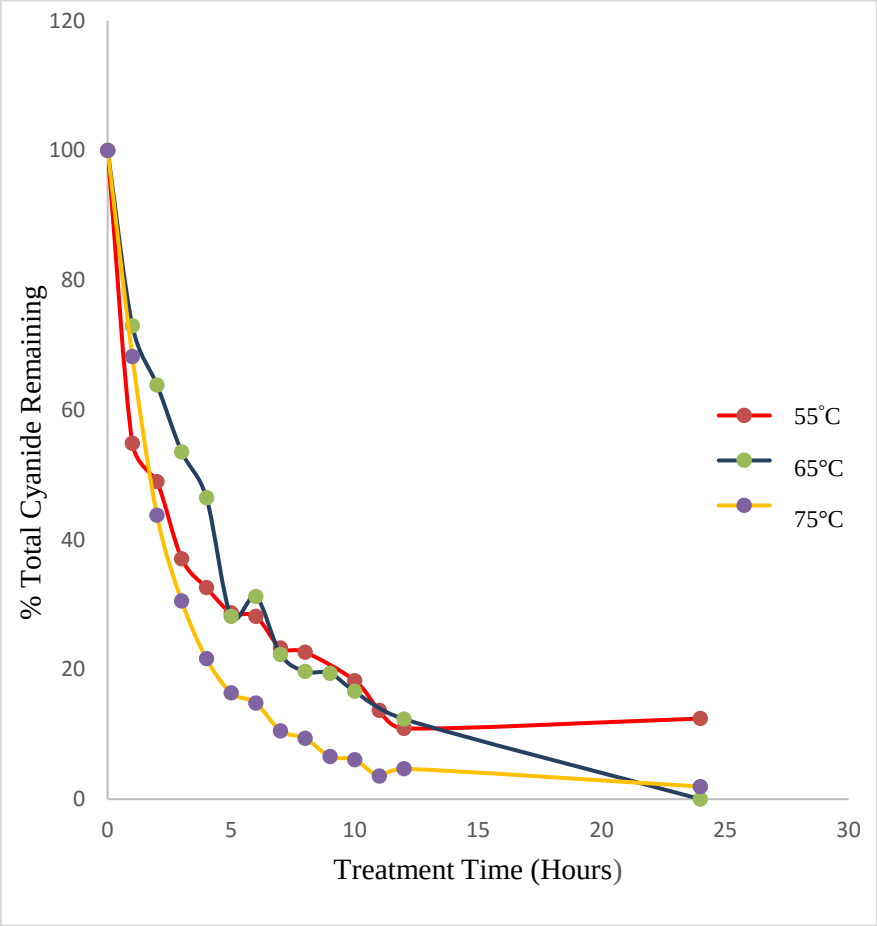


Figure 4-2. The effect of time and increased temperature on the ability of the SWM to reduce the % total cyanide remaining (TCR) in flaxseed flour. Ground flaxseed flour (5 g) was mixed with 10 g water in a 100-ml beaker. The replicates were performed on separate days to determine the between experimental error. Each line represents the average of three replicates performed on different days at the same temperature.

Graphically, the decline in %TCR is distinguished by three time intervals: From 0 h to 6 h a rapid decline in TCR was observed at all three temperatures, with the 75˚C treatment showing greatest decrease in TCR (94% reduction). From 6 h to 10 h TCR at all temperatures continued to decrease but at lower rates. From 10 h to 24 h TCR rates of decline showed more variation. The 75˚C treatment had nearly obtained its minimum value (1.9% TCR) at the beginning of this interval, whereas an observable TCR decline in the 65˚C treatment continued to 24 h (<1% TCR) but was not present at 55˚C treatment (12% TCR).

All four temperatures were compared in terms of the time required to achieve 17% TCR (6-fold reduction) and TCR at 24 h (Table 4-1).

Table 4-1. A comparison of all three temperatures in terms of time required to achieve 17% TCR and TCR at 24 h. Temperatures below 75˚C were not significantly different in their time required to achieve 17% (6-fold reduction) TCR. At 75˚C, 17% TCR was achieved in approximately half the time (4.9 h verses 9.9 averaged h). At 24 h, all treatment temperatures had achieved below 5% TCR, with the exception of 55˚C (possibly due to measurement error).

Treatment Temperature (˚C)	Time for 17% TCR (h)	TCR at 24 h (%)
22	10	4.0
55	9.9	12.4
65	9.8	<1
75	4.9	1.9

Based on the data above, a SWM treatment of 12 h at 75˚C was used to examine the effect of treatment vessel geometry on %TCR (Table 4-2 and Fig. 4-3). Extended treatment time effects were also determined (Replicate 1).

Table 4-2. The effects of three different exposed surface areas on the reduction of % total cyanide remaining (TCR) after application of the SWM at 75˚C for extended processing times (h) on three different days (replicates 1-3). Tabulated values reflect %TCR of averaged triplicate measurements. At 12 h, average TCR for thin (0.5 cm), thick (0.2), and 10 cm depths was respectively 6.0%, 35.2%, and 54.1%. Average values indicate thin (0.5 cm) depths had

approximately 6 times greater TCR reduction than thick (0.2 cm) and 9 times greater reduction in TCR than 10 cm depths.

Hours	Thin (0.5cm)	Thick (2 cm)	Cylinder (10 cm)	Thin (0.5cm)	Thick (2 cm)	Cylinder (10 cm)	Thin (0.5cm)	Thick (2 cm)	Cylinder (10 cm)
	Replicate 1			Replicate 2			Replicate 3		
0	100	100	100	100	100	100	100	100	100
2	52.5	75.6	81	61.6	70.5	71.7	58.5	73.8	57
4	37.6	71.3	69.4	53.9	89.4	90.1	49.3	63.4	71.2
6	ND	ND	ND	33	71.8	94	24.6	55.5	73.3
8	20	61.4	76.1	19.7	66.8	77.9	13.9	58	55.9
10	ND	ND	ND	ND	ND	ND	4.7	38.3	66
12	7.7	30.8	50.3	8	42.1	56.3	2.3	32.8	55.7
24	1.6	20.7	40.4	ND	ND	ND	ND	ND	ND

ND = Not Determined

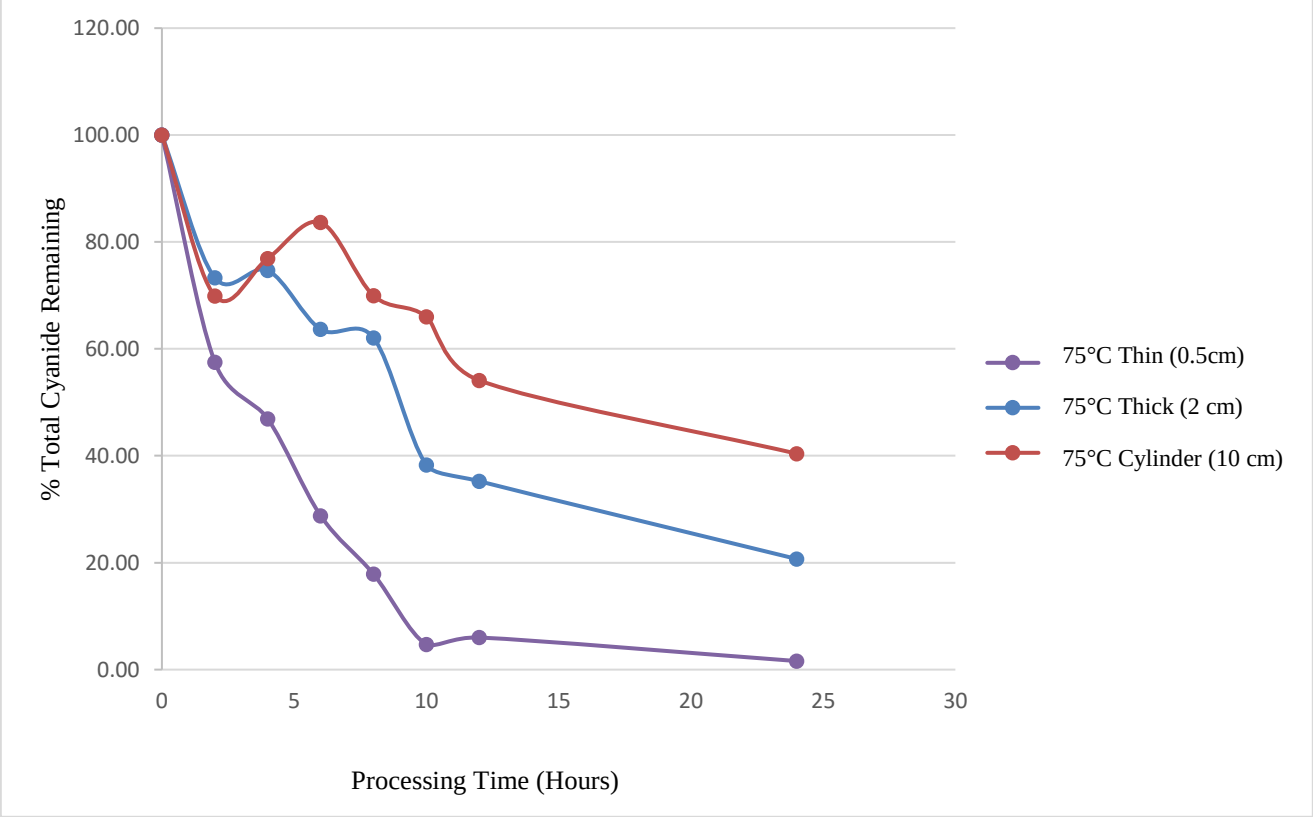


Figure 4-3. Effect of increased exposed SA (represented by differential thickness depths) on % TCR, achieved by spreading 250 grams flaxseed flour wetted with 500 grams water to various thicknesses (0.5 cm, 2 cm, or 10 cm) and subsequent heating at 75˚C for a 12 hour processing time and beyond. Each line represents the average of three replicates performed on different days at the same SA referenced by its thickness.

For thin (0.5 cm) depths, 17% (6-fold) TCR occurred after approximately 8 h and was not achieved by the thicker depths at 24 h. At 12 h optimal treatment time, TCR for thin (0.5 cm), thick (2 cm), and 10 cm depths was respectively 6.0%, 35.2%, and 54.1%. Thus, the thin (0.5 cm) depths resulted in 9 times the reduction rate of (10 cm) depths. At 24 h extended treatment time, TCR for thin (0.5 cm), thick (2 cm), and 10 cm depths was 1.6%, 20.7%, and 40.4%, respectively. Treatment time extension to 24 h lowered the TCR for the thin (0.5 cm) depth by an additional 4.2 % (both low values below measurement sensitivity); thicker depths retained 20.7% and 40.4% TCR, respectively for the thick (2 cm) and 10 cm depths. Thicker depths (2 cm and 10 cm) displayed inconsistent rates of TCR decline, possibly due to sampling at differential depths with greater HCN retention at lower depths.

The effect of exposed surface area on the efficiency of the SWM to reduce the % TCR in the flax flour was also investigated at the lower temperature of 22°C. The results were recorded in Table 4.3 and presented in Figures 4-4 and 4-5.

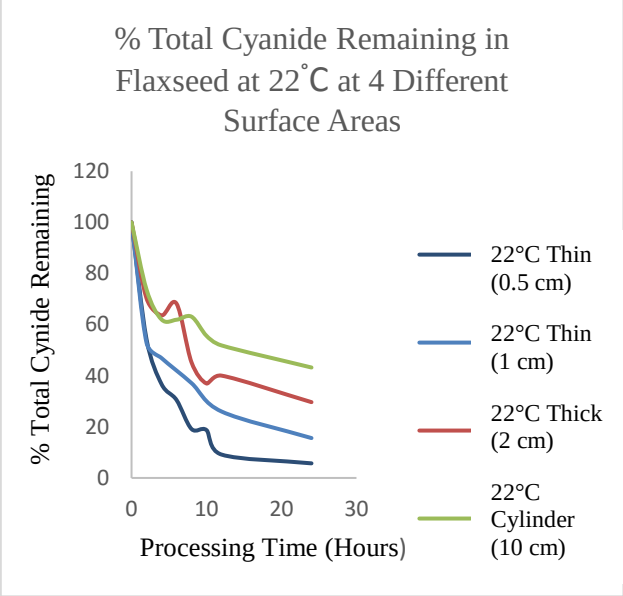
At 12 h, average TCR for thin (0.5 cm), thick (0.2), and 10 cm depths was respectively 9.2%, 40.0%, and 51.9%. Average values indicate thin (0.5 cm) depths had approximately 4.4 times greater TCR reduction than thick (0.2 cm) and 5.6 times greater than 10 cm depths. At 24 h, average TCR for thin (0.5 cm), thick (0.2), and 10 cm depths was respectively 9.0%, 29.0%, and 43.2%. Average 24 h values indicate thin (0.5 cm) depths had approximately 3.2 times greater TCR reduction than thick (0.2 cm) and 4.8 times greater than 10 cm depths. The (0.5 cm) thin depths had approximately three times less TCR than the thin (1 cm) depths (5.7% average compared to 15.6%).

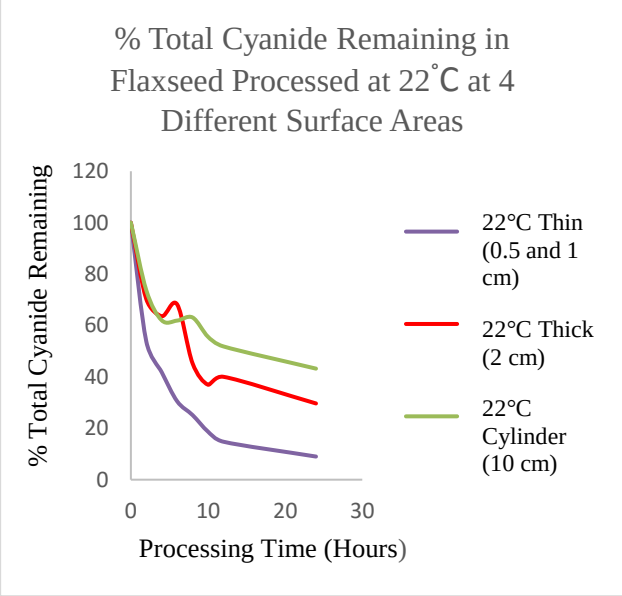
Table 4-3. The effects of three different treatment vessel geometries on the reduction of % total cyanide remaining (TCR) after application of the SWM at 22°C for extended processing times (h) on three different days (replicates1-3). Tabulated values reflect %TCR of averaged triplicate

measurements (Note that the thin depth of replicate 1 is 1 cm, twice the thickness of the subsequent thin SAs).

Hours	Thin (1cm)	Thick (2 cm)	Cylinder (10 cm)	Thin (0.5cm)	Thick (2 cm)	Thin (0.5cm)	Thick (2 cm)	Cylinder (10 cm)
	Replicate 1			Replicate 2		Replicate 3		
0	100	100	100	100	100	100	100	100
2	52.9	73.3	68	52.8	67.1	55.7	70.4	79.4
4	46.9	58.1	61.8	NA	59.3	36.7	73.4	62.2
6	NA	NA	NA	NA	NA	30.5	68.2	61.9
8	37.1	42.4	64.1	12.9	42.1	25.4	51.2	62.1
10	NA	NA	NA	NA	NA	18.7	46.4	55.6
12	26	51.4	56.3	2.9	27.5	15.4	41	47.4
24	15.6	32.3	51.4	5.6	27.7	5.8	27	35

ND = Not Determined





Figures 4-4 and 4-5. Effect of increased exposed SA (represented by differential thickness depths) on % TCR, achieved by spreading 750 g of wetted flaxseed flour mixture to various thicknesses (0.5 cm, 1 cm, 2 cm, or 10 cm) at 22˚C for a 24 h processing time. Each line represents the average of three replicates performed on different days at the same SA referenced by its thickness. In Figure 4-4, the thin depths (0.5 cm and 1 cm) are graphed separately, whereas in Figure 4-5, the thin depths (0.5 cm and 1 cm) are combined. This was done to gauge the sensitivity of the thin depth.

For thin (0.5 cm) depths, 17% (6-fold) TCR occurred after approximately 8-10 h and was not achieved by the thicker depths at 24 h. For thin (1 cm) depths, 17% (6-fold) TCR occurred after approximately 21 h and was not achieved by the thicker depths at 24 h. Thin (0.5 cm) thickness had 2.6 times the reduction rate of the thin (1 cm) thickness.

At 24 h extended treatment time, TCR for thin (0.5 cm), thin (1 cm), thick (2 cm), and 10 cm depths was 5.7%, 15.6%, 29.37%, and 43.2%, respectively. Treatment time extension to 24 h resulted in reduction of TCR by 38% between extremes (0.5 cm compared to 10 cm depth). Thicker depths (2 cm and 10 cm) displayed inconsistent rates of TCR decline, possibly due to sampling at differential depths with greater HCN retention at lower depths.

With the aid of statistical analysis using general linear mixed models (LMMs), an optimal SWM for flaxseed flour aimed toward efficient consumer utilization was determined from this data. For flaxseed flour processed at 75˚C, SA was shown to be a significant factor in minimizing the %TCR (Chi-square = 37.35, df = 2, $p < 0.0001$). %TCR was significantly reduced over time (Chi-square = 59.776, df = 1, $p < 0.001$), and the pairwise comparisons for the SA were as follows:

1) %TCR measurements were not significantly different for flaxseed processed in a thick (2 cm) depth compared to flaxseed processed at 10 cm thickness ($t = 1.8176$, $p = 0.0753$).
2) %TCR measurements were significantly lower for flaxseed processed in a thin (0.5 cm) depth compared to flaxseed processed at 10 cm thickness ($t = 7.060$, $p < 0.0001$).
3) %TCR measurements were significantly lower for flaxseed processed in a thin (0.5 cm) depth compared to flaxseed processed at a thick (2 cm depth) ($t = 5.242$, $p < 0.0001$).

For flaxseed flour processed at 22˚C, SA was shown to be a significant factor in minimizing the %TCR (Chi-square = 19.903, df = 2, $p < 0.0001$). %TCR is significantly reduced over time (Chi-square = 50.293, df = 1, $p < 0.0001$), and the pairwise comparisons for the SA are as follows:

1) %TCR measurements were not significantly different for flaxseed processed in a thick (2 cm) depth compared to flaxseed processed at 10 cm thickness ($t = 1.237$, $p = 0.226$).

2) %TCR measurements were significantly lower for flaxseed processed in a thin (0.5 cm) depth compared to flaxseed processed at 10 cm thickness ($t = 4.588$, $p < 0.0001$).
3) %TCR measurements were significantly lower for flaxseed processed in a thin (0.5 cm) depth compared to flaxseed processed at a thick (2 cm depth) ($t = 3.699$, $p = 0.006$).

Thin (0.5 cm) SA representations were selected as optimal for reducing TCR at both 75˚C and 22˚C. A final analysis compared ambient temperature (previously analyzed separately as a potentially more energy efficient stand-alone treatment option) to 75˚C treatment, each processed under thin exposed SA representations for extended treatment times (Fig 4-6). Once the variable of increased exposed SA (represented by the thin 0.5 cm thickness) was added to the analysis, the treatment temperatures of 22˚C and 75˚C were no longer significantly different ($p = 0.871566$).

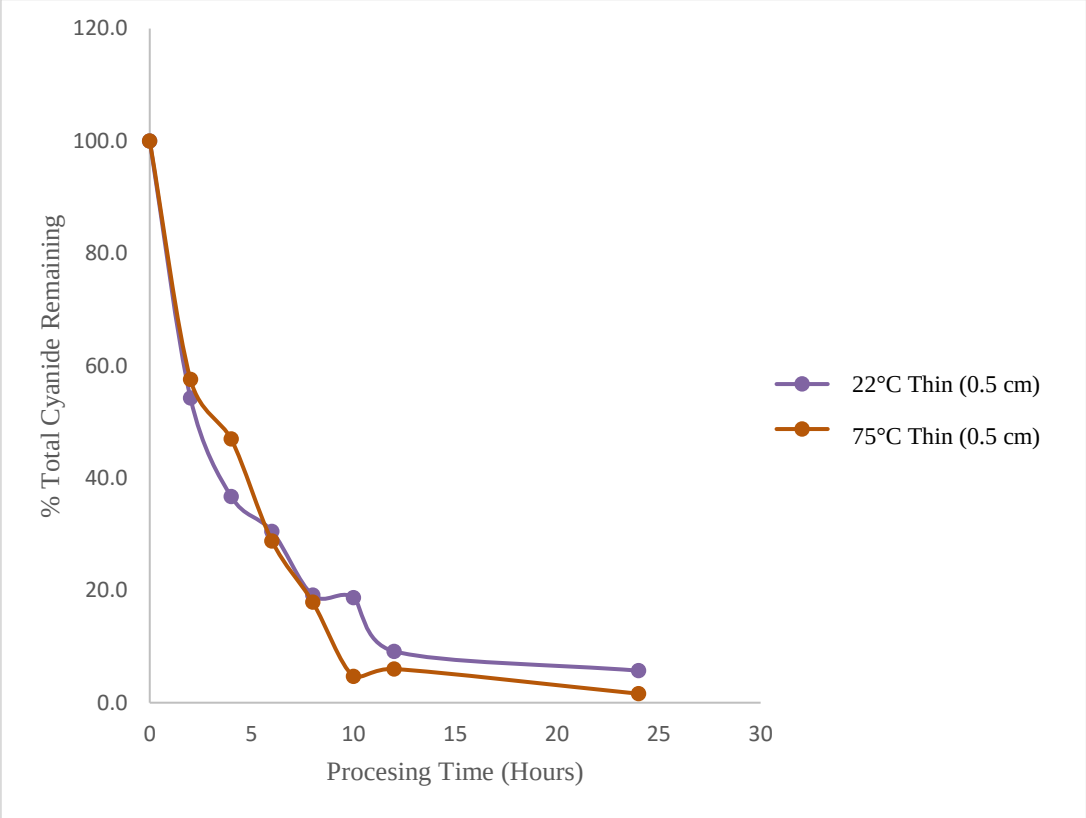


Figure 4-6. Comparison of flaxseed processed with SWM in thin (0.5) depths at 22°C and 75°C over time. Added exposed SA effect (represented by thin 0.5 cm depth) appeared to negate

temperature dependence of TCR at 75˚C and 22˚C; treatment temperatures were no longer significantly different (p = 0.871566).

Therefore, an optimal SWM aimed toward consumer utilization was designed for flaxseed flour in terms of time, temperature and exposed SA for maximum reduction within measurement sensitivity of TCR and determined to be 12 h, 22˚C, with 0.5 cm thickness (resulting in 9.2 %TCR). For a nearly 6-fold reduction, an efficient reduction in TCR was determined as 8 h, 22˚C, and 0.5 cm thickness (with 19.1 %TCR).

4.2. Retention of SDG

HPLC analysis of the lignan SDG showed that the SWM had little effect on this desirable compound, especially when processed at ambient temperature (91.8% - 98.9% retention), but also when processed at 75°C (77.3% - 93.2% retention). The concentration of SDG measured ranged from 2.72 mg/g to 3.52 mg/g flaxseed flour.

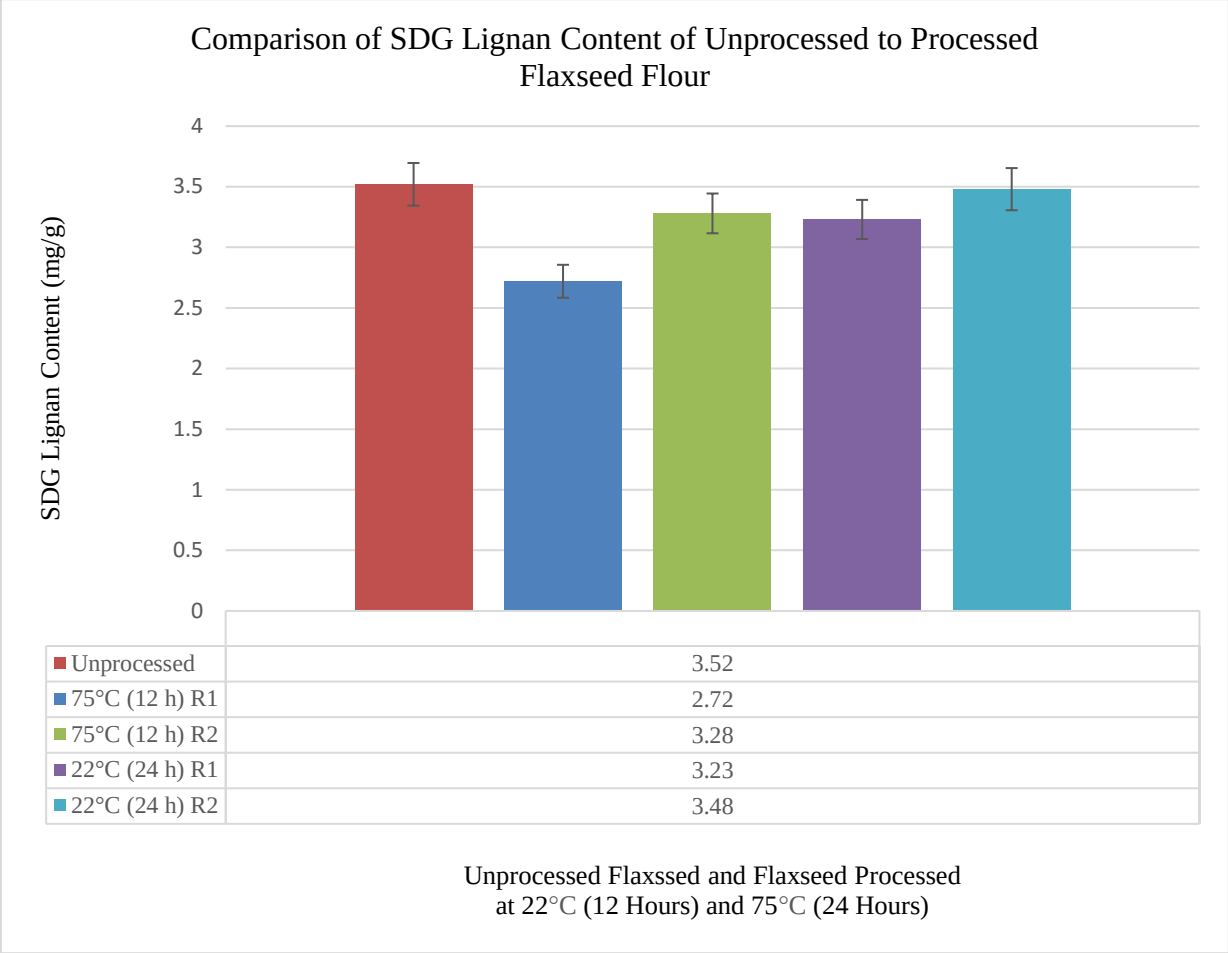


Figure 4-7. Comparison of secoisolariciresinol diglucoside (SDG) lignan content (mg/g) between unprocessed flaxseed flour (left) and flaxseed flour processed by the SWM at 22˚C for 24 hours (the two right most bars) and flaxseed flour processed at 75˚C for 12 hours (second and third bars). The wetted flaxseed flour was either analyzed directly (before processing) or it was analyzed after processing by the optimal SWM for flaxseed flour (75˚C, 12 hours, 0.5 cm thickness), or after extended processing at ambient temperature (22˚C, 24 hours, 0.5 cm thickness). The flaxseed flour processed at 75˚C retained between 77.27% (replicate 1) and 93.18% (replicate 2) of the original SDG lignan content. The flaxseed flour processed at extended ambient temperature (22˚C) retained between 91.76% (replicate 1) and 98.86% (replicate 2) of the original SDG lignan content. The analytical method involved extraction of ground wetted flaxseed with dioxane/ethanol, aqueous base-hydrolysis, solid-phase purification of and SDG fraction, and quantification by high performance liquid chromatography (HPLC) as described by Johnsson et al (2000).

4.3. Other Measurements

The pH was monitored at completion of the experiments and the termination time points typically had a pH above 6, with the lowest recording at pH 5.9.

The *B*-glucosidase Assay for endogenous *B*-glucosidase enzymes (linamarase and linustatinase) indicated that mean level of endogenous enzyme activity measurement was above 250 units/L, indicating a very high enzyme activity.

The results of the plate count showed that no substantial growth (3.14×10^6 CFU) appeared on the flaxseed flour processed by the optimal SWM (12 h, 22˚C, 0.5cm depth) when compared to the unprocessed flaxseed flour at time 0 (3.09×10^6 CFU).

5. Discussion

5.1 Total Cyanogen Degradation

The current project has demonstrated that the SWM developed by Bradbury and co-workers (Bradbury, 2006) for the removal of cyanogenic glycosides from cassava is equally efficacious for the removal of cyanogens from flaxseed. In previous cassava studies, a 6-fold reduction in TCR was achieved in 5 h through applying the SWM with heat (55˚C) and spreading the wetted mixture out into 0.5 cm layers (Bradbury and Denton, 2010). However, in the current study, simultaneous application of elevated temperature (75˚C) and similar spreading technique demonstrated that temperature influence on TCR reduction was no longer significant; thin (0.5 cm) depths resulted in TCR degradation curves that were not significantly different at 22˚C than 75˚C with a nearly 6-fold reduction in TCR measured in approximately 8 hours for each (80.9% and 82.1% total cyanide removed, respectively) (Fig. 4-6). The lack of temperature effect when combined with the increased exposed SA of the thin (0.5) depth may be due to the significantly higher oil content in flaxseed (41%) than cassava (< 1%). Excessive oil may hinder the ability of the water-soluble HCN to volatilize, hence the increase in exposed SA achieved by spreading wetted material into thin (0.5

cm) depths may be more necessary to facilitate HCN volatilization in flaxseed than cassava and may, therefore, outweigh temperature influence on %TCR.

Other methods currently developed to remove cyanide from flaxseed were able to remove a higher percentage of total cyanogens (Wanasundra et al., 1993; Wu et al., 2012; Yamashita et al., 2006). A solvent extraction method was developed that reported removal of over 90% of the cyanogenic glycosides in flaxseed (removal of total cyanogens was not discussed), but it also removes some of the beneficial components (Wanasundra et al., 1993; and Yamashita et al., 2006). Moreover, concern exists regarding the use of solvent extraction in food processing, both in terms of environmental degradation and human health. Other more recent fermentation methods have been developed that remove up to the detection limit or up to 99.3% of the total cyanogens (Wu et al., 2012; Yamashita et al., 2006). The method by Yamashita involved incubation at 30˚C for 18 h followed by an energy consumptive steam evaporation at 120˚C which could remove 1 kg per h on a conveyor belt in a steam room (Yamashita et al., 2006). The method by Wu involved powdering the flaxseed in a disintegrator and fermenting it for 48 h at 46.8˚C with an enzyme preparation that includes human liver β-glucosidase and *Bacillus* sp. cyanide hydratase produced by a genetically modified *Pichia* strain (making it unacceptable to certain consumers).

The SWM investigated in this study was able to remove 90.8% of the total cyanogens after a treatment time of 12 h at 22˚C when spread to a thin depth of 0.5 cm. Though this SWM removes approximately 10% less total cyanogens than some of above mentioned methods, it has the advantage of being more acceptable, less cost and energy consumptive, and simpler. Also, the cyanogenic levels in flaxseed are low when compared to plants like cassava and it is not necessary to completely remove the cyanogenic glycosides in order to maximize the potential value of this

food source. A greater concern regarding the utility of the SWM applied to flaxseed would be the nature of the residual cyanogens; if the more toxic cyanohydrin intermediates remain, then the method would be less successful. Nevertheless, the flaxseed flour would still net an overall distinct reduction in TCR after processing according to the optimal SWM procedure.

The current study examined the effects of three variables on the effectiveness of the SWM when applied to flaxseed flour: extended processing time, temperature elevation, and the effects of increased exposed SAs (represented by spreading 750 g wetted flax mixtures to diverse thickness). Extending the processing time to 24 hours resulted in a continual total cyanide loss for the flaxseed processed at 22˚C. Temperature elevation was shown to increase the rate of % total cyanide reduction in the flaxseed flour (Fig. 4-2). This was presumably because temperature influences the rate of breakdown of the cyanogenic glycosides by the endogenous *B*-glucosidase enzymes (linamarase and linustatinase), measured in this study to have a very high activity of > 250 units/L.

Increasing the SA of the flaxseed flour mixture by spreading it out into thin layers also significantly reduced the %TCR. General linear mixed model (LMMs) analysis was used to determine the significance of time and increased exposed SA (inversely represented by thicknesses) in the removal of cyanogenic compounds from flaxseed flour. For flaxseed flour processed at 75˚C, SA was shown to be a significant factor in minimizing the %TCR (Chi-square = 37.35, df = 2, $p < 0.0001$) with the thin (0.5 cm) depths having the most effect ($t = 5.242$, $p < 0.0001$). Pairwise comparison for the different thicknesses used to evaluate SA effects of the SWM additionally showed that the %TCR was not significantly different for flaxseed processed at thick 2 cm depths compared to flaxseed processed at 10 cm depths (cylinder container) (($t = 1.8176$, $p = 0.0753$). Time was also a significant factor in minimizing %TCR at this temperature (Chi-square = 59.776,

df = 1, $p < 0.0001$). For flaxseed flour processed 22°C, SA was also shown to be a significant factor in minimizing the %TCR (Chi-square = 19.903, df = 2, $p < 0.0001$) with the thin depths (0.5cm) having the most effect ($t = 3.699$, $p = 0.006$). Pairwise comparison for the different thicknesses used to evaluate SA effects of the simple wetting again showed that the %TCR was not significantly different for flaxseed processed at thick (2 cm) depths compared to flaxseed processed at 10 cm thickness (cylinder container) ($t= 1.237$, $p = 0.226$). Time was also a significant factor in minimizing %TCR at this temperature (Chi-square = 50.293, df = 1, $p < 0.0001$). Clearly, an increase in exposed SA facilitated the removal of the water-soluble HCN gas in the damp flour mixtures (Figures 4-3 through 4-5, Tables 4-2 and 4-3). This finding was consistent with the analysis of cassava flour where a 500 g wetted material spread out thin (0.5 cm) for 5 h at 30°C resulted in 18% TCR, whereas retention was 38% TCR for the samples processed similarly at 4 cm depth (Cumbana et al., 2007).

The optimal parameters selected for application of the SWM for flaxseed flour were: 12 h, 22°C, and 0.5 cm thickness for maximum reduction in TCR at the sensitivity level of the measurement technique. Flaxseed flour processed under these conditions averaged a reduction of 90.8 % total cyanogens (or 9.2 %TCR). However, the picrate method used to measure the total cyanogens remaining in the flaxseed flour is less sensitive when measuring the lower ppm values in the range of 10% TCR, and this needs to be kept in mind when interpreting the data. A more sensitive picrate method has been developed that might aid in providing more accurate measurements of TCR for future investigations in these very low ranges (Bradbury, 2009).

At both 22°C and 75°C, there was less agreement among the results of the independent trials at thicker depths, especially of the 10 cm depth. At 75°C, the correlation between thin SA depths was

high ($r^2 = 0.99$), it was lower between thick SA depths ($r^2 = 0.93$), and was depressed between the 10 cm SA layers ($r^2 = 0.78$). This was attributed to the HCN becoming "trapped" in the dough reducing its ability to volatilize (Cumbana, 2007). Thus, when sampling from the thick layers (2cm and 10 cm) of the flaxseed mixture, samples were taken at various depths of the HCN entrapped dough, which was reflected by a greater diversity in %TCR remaining (leading to less correlation between samples at greater thicknesses when compared to thin).

The inconsistency between sampling days may also be due to the differential moisture content in the flaxseed mixture between sampling. Recall that to initiate the wetting method, the flaxseed flour is mixed with water at a hydration level of 1 part flaxseed flour to 2 parts water (Bradbury, 2006). After hydrating the ground flaxseed samples, the water present in the flaxseed mixture rapidly swells the flaxseed particles, bringing the hydrolytic enzymes into contact with cyanogenic glycosides, allowing hydrolysis to occur. Therefore, it was important not to let the samples dry-out over the time course of the experiment, or the rate of hydrolysis would decrease. Care was taken to monitor the samples, and if required water was replenishing at intervals during the experimental procedure. However, the samples were sometimes replenished just prior to sampling (when the evaporative water loss needed to be corrected) and, therefore, the flaxseed flour processed at higher temperatures had more variability in hydration throughout the experimental time course.
This "drying out" may be reflective of what might happen in actual practice upon household application of the SWM to flaxseed flour, especially if applying heat. Therefore, the fact that the method still works (albeit, most likely at a reduced rate) after the flour goes through periods of drying and subsequent rehydration, is noteworthy. The method could potentially be significantly improved through incorporating a mechanism of keeping the mixture hydrated, such as through frequent misting. The effect of "flooding" the dough during the treatment period was not

investigated, and this too may be a potential way to decrease the treatment time required to minimize the TCR.

Also, it is important to recognize that the SWM is successful at significantly reducing the TCR in flaxseed flour processed at the lower temperature of 22˚C. This is the first known research that has implemented the SWM at temperatures lower than 30˚C, and these findings, therefore, fill a research need (Bradbury, 2006). Furthermore, it was claimed that the spontaneous breakdown of the acetone cyanohydrin occurs at temperatures above 30˚C (Vetter, 1999), yet here it is found that the total cyanogens in the flaxseed have been significantly reduced at 22˚C. Future research can evaluate the nature of the cyanogenic compound(s) (cyanogenic glycoside, cyanohydrin, or HCN), remaining in the processed flaxseed flour (if any). This knowledge would also help satisfy the need stated by the WHO for more data showing the ratio of cyanogenic glycosides to cyanohydrin to HCN in raw and processed versions of a wide range of foods containing cyanogenic glycosides (WHO, 2012).

5.2. Lignan retention

The results of the comparative analysis of the lignan SDG content in the pre and post-processed samples showed that the SWM used to significantly degrade TCR in flaxseed flour did not also considerably reduce the desirable lignan component. The flaxseed processed by the SWM at 75˚C for 12 h at the thin (0.5 cm) depth showed the greatest loss in SDG content, ranging from a 77.3% to 93.2 % recovery. This difference was considered partially due to measurement error, as the method proved to be challenging to perform on non-defatted flaxseed. The concentration of SDG in the unprocessed flaxseed and the flaxseed processed under ambient conditions at extended treatment times (22˚C, 24 h, thin 0.5 cm depths) was not significantly different, measuring 91.8% to

98.9 % recovery. However, the determined concentration of SDG in the flaxseed, unprocessed or not, was lower than expected. This was attributed to the fact that the flaxseed was wetted according to the SWM protocol prior to extraction with an ethanol/dioxane solvent for the first step in preparation of samples for HPLC analysis. The resultant decrease in miscibility may explain the low SDG yield. However, since all samples were prepared similarly and the outcome measure was relative, the lower than expected SDG concentrations would not be expected to influence the conclusion of overall positive SDG retention after SWM processing.

This finding is consistent with other research investigating the effects of processing on flaxseed. The fermentative processing techniques discussed earlier by Yamashita and Wu involved incubating wetted flaxseed meal (i.e., defatted flaxseed) for 18 and 48 h at 30-46.8˚C, respectively, yet the lignan content was not diminished in either study (Yamashita et al., 2005, Wu et al., 2012). Furthermore, other investigations into the effect of thermal heating on lignans have found that heating at 250˚C over normal baking periods did not degrade flaxseed SDG lignans (Gerstenmeyer et al., 2013), and therefore heating at heating at 75˚C may not degrade the lignans even over a much longer time period.

The retention of lignans after processing is important because flaxseed, by far the richest known plant source of the lignan SDG, is a food with tremendous nutritional potential. It is considered a functional food as its consumption may enhance health or prevent disease; studies indicate that flaxseed protects against the development of chronic diseases such as CVD, hormone-sensitive cancers, and diabetes, with much of this health benefit attributed to the SDG lignan content of flax. Yet, research suggests that doses of 500 mg SDG/day are needed to observe maximum health gain (Cunnane et al., 1995; Lucus et al., 2002). A level of 500 mg SDG corresponds to approximately 38-82 grams whole flaxseeds (Hallund et al., 2006; Johnsson et al., 2000). In order to ingest this

amount of SDG from flax daily, approximately 4 times the recommended safe level of flaxseed (10-20 grams) (Strandas, 2008) would need to be consumed.

Currently, whole flaxseed available in markets for human consumption is sold untreated, and so it includes the cyanogenic glycoside toxicants. However, if the flaxseed were processed by household cooks utilizing the SWM, it might be possible to safely consume flaxseed at more protective levels; this could easily be achieved by eating a small bowl of ground flaxseed as cereal.

The bioavailability of flaxseeds is enhanced when they are milled. Therefore it is preferable to utilize them as a ground flour for food products (Kuijsten et al., 2005); studies have also indicated that consumption of whole flax foods may provide synergistic health benefits not found in individual flax components (Goyal et al., 2014; Hallund et al., 2006; Oomah, 2001; Styrczewska et al., 2013). Therefore, a strength of the SWM is it provides a whole food source of ground flax that has been processed to remove cyanogenic toxicants by a method that is more acceptable, cost and energy efficient, and simpler than existing methods. A limitation of this method however, is that it may be ineffective for certain processed foods such as heat-pressed flaxseed meal or flour that no longer contains active endogenous β-glucosidase enzymes.

6. Conclusions

A SWM used to significantly reduce the % total cyanide remaining in cassava flour was evaluated for its applicability to ground flaxseed. An optimal SWM in terms of time, temperature, and exposed SA depth was developed by spreading the flaxseed out in a thin depth of 0.5 cm (which enables the HCN to volatilize). This method resulted in 90.8 % removal of total cyanogens from flaxseed flour. General mixed model analysis confirmed that the variables time and SA were significant factors in minimizing the %TCR in the optimal model. Pairwise comparison showed

that the thin (0.5 cm) depth had the greatest effect on reducing the %TCR in flaxseed. The optimal parameters for the SWM aimed at efficient consumer utilization of flaxseed were a 12 h treatment time, a temperature of 22˚C, and an increased exposed SA achieved by spreading the wetted mixture out to a thin depth of 0.5 cm.

This research project is significant in that it confirmed the ability of a SWM to reduce the cyanide content in ground flaxseed so that more of this functional food may safely be consumed. The goal of this research is to initiate a pilot project providing a technique to be used on a household level for removing significant quantities of total cyanogens from ground flaxseeds. Future research utilizing more sensitive analysis can confirm these finding and evaluate the nature of any cyanogenic compound(s) (cyanogenic glycoside, cyanohydrin, or HCN), remaining in the processed flaxseed flour.

7. Appendix I

The following code was written in R for the determination of LMMs (m.all, m.hrs, m.sa) 22 degrees:

```
> #set working directory - where files are stored
> setwd('C:/Users/Sandra/Desktop')
>
> #import packages necessary for analyses
> library(tidyr)
> library(lme4)
> library(pbkrtest)
>
> #import data
> dat <- read.csv('thesis data 22 degrees.csv',header=T)
> # dat <- read.csv('thesis data 75 degrees.csv',header=T)
> dat <- gather(dat,surface.area,tcr,thin:beaker)
> dat$surface.area <- as.factor(dat$surface.area)
>
> #run models
> m.all <- lmer(tcr~hrs+surface.area+(1|experiment),data=dat,REML=FALSE)
> m.hrs <- lmer(tcr~hrs+(1|experiment),data=dat,REML=FALSE)
> m.sa <- lmer(tcr~surface.area+(1|experiment),data=dat,REML=FALSE)
>
> #model comparisons
> anova(m.all,m.hrs) #tests whether the surface area factor is significant
Data: dat
Models:
m.hrs: tcr ~ hrs + (1 | experiment)
m.all: tcr ~ hrs + surface.area + (1 | experiment)
     Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
m.hrs  4 468.84 476.72 -230.42   460.84
m.all  6 452.93 464.76 -220.47   440.93 19.903      2  4.766e-05 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
> anova(m.all,m.sa) #tests whether the hrs factor is significant
Data: dat
Models:
m.sa: tcr ~ surface.area + (1 | experiment)
m.all: tcr ~ hrs + surface.area + (1 | experiment)
     Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
m.sa   5 501.23 511.08 -245.61   491.23
m.all  6 452.93 464.76 -220.47   440.93 50.293      1  1.324e-12 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
> #get p values (using Kenward-Roger approximation)
> coefs <- data.frame(coef(summary(m.all)))
> df.KR <- get_ddf_Lb(m.all,fixef(m.all))
> coefs$p.KR <- 2*(1-pt(abs(coefs$t.value),df.KR))
> coefs
                  Estimate Std..Error   t.value         p.KR
(Intercept)       85.771142  4.7406299 18.092773 0.000000e+00
hrs               -2.548241  0.2782103 -9.159407 4.982708e-10
surface.areathick  -6.680739  5.4010745 -1.236928 2.261099e-01
surface.areathin  -25.049295  5.4596953 -4.588039 8.078322e-05
```

```
>
> #re-depth surface area & re-run model to get all pairwise comparisons
> dat$surface.area <- redepth(dat$surface.area,ref="thin")
> m.all2 <- lmer(tcr~hrs+surface.area+(1|experiment),data=dat,REML=FALSE)
> summary(m.all2)
Linear mixed model fit by maximum likelihood  ['lmerMod']
Formula: tcr ~ hrs + surface.area + (1 | experiment)
   Data: dat

     AIC      BIC   logLik deviance df.resid
   452.9    464.8   -220.5    440.9       47

Scaled residuals:
    Min      1Q  Median      3Q     Max
-1.7701 -0.6966 -0.2088  0.5850  2.5341

Random effects:
 Groups     Name        Variance Std.Dev.
 experiment (Intercept)   0.0     0.0
 Residual              240.2    15.5
Number of obs: 53, groups:  experiment, 3

Fixed effects:
                 Estimate Std. Error t value
(Intercept)       60.7218     4.2745  14.206
hrs               -2.5482     0.2782  -9.159
surface.areabeaker  25.0493     5.4597   4.588
surface.areathick   18.3686     4.9659   3.699

Correlation of Fixed Effects:
            (Intr) hrs    srfc.rb
hrs         -0.555
surfac.rbkr -0.549  0.012
surfc.rthck -0.603  0.013  0.467
>
> coefs <- data.frame(coef(summary(m.all2)))
> df.KR <- get_ddf_Lb(m.all2,fixef(m.all2))
> coefs$p.KR <- 2*(1-pt(abs(coefs$t.value),df.KR))
> coefs
                  Estimate Std..Error   t.value         p.KR
(Intercept)       60.721847  4.2744511 14.205765 5.006539e-07
hrs               -2.548241  0.2782103 -9.159407 1.462342e-05
surface.areabeaker 25.049295  5.4596953  4.588039 1.708794e-03
surface.areathick  18.368556  4.9658664  3.698963 5.879969e-03a



>
```

75 degrees:

```
> #set working directory - where files are stored
> setwd('C:/Users/Sandra/Desktop')
>
> #import packages necessary for analyses
> library(tidyr)
> library(lme4)
> library(pbkrtest)
>
> #import data
> # dat <- read.csv('thesis data 22 degrees.csv',header=T)
> dat <- read.csv('thesis data 75 degrees.csv',header=T)
> dat <- gather(dat,surface.area,tcr,thin:beaker)
> dat$surface.area <- as.factor(dat$surface.area)
>
> #run models
> m.all <- lmer(tcr~hrs+surface.area+(1|experiment),data=dat,REML=FALSE)
> m.hrs <- lmer(tcr~hrs+(1|experiment),data=dat,REML=FALSE)
> m.sa <- lmer(tcr~surface.area+(1|experiment),data=dat,REML=FALSE)
>
> #model comparisons
> anova(m.all,m.hrs) #tests whether the surface area factor is significant
Data: dat
Models:
m.hrs: tcr ~ hrs + (1 | experiment)
m.all: tcr ~ hrs + surface.area + (1 | experiment)
      Df    AIC    BIC  logLik deviance Chisq Chi Df Pr(>Chisq)
m.hrs  4 514.81 522.98 -253.41   506.81
m.all  6 481.46 493.72 -234.73   469.46 37.35      2  7.756e-09 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
> anova(m.all,m.sa) #tests whether the hrs factor is significant
Data: dat
Models:
m.sa: tcr ~ surface.area + (1 | experiment)
m.all: tcr ~ hrs + surface.area + (1 | experiment)
      Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
m.sa   5 539.24 549.45 -264.62   529.24
m.all  6 481.46 493.72 -234.73   469.46 59.776      1  1.063e-14 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
> #get p values (using Kenward-Roger approximation)
> coefs <- data.frame(coef(summary(m.all)))
> df.KR <- get_ddf_Lb(m.all,fixef(m.all))
> coefs$p.KR <- 2*(1-pt(abs(coefs$t.value),df.KR))
> coefs
                  Estimate Std..Error    t.value         p.KR
(Intercept)       96.234081  4.3821035  21.960705 0.000000e+00
hrs               -3.543669  0.3423596 -10.350723 7.993606e-14
surface.areathick  -8.636842  4.7516317  -1.817658 7.535432e-02
surface.areathin  -33.547368  4.7516317  -7.060179 5.914738e-09
>
> #re-depth surface area & re-run model to get all pairwise comparisons
> dat$surface.area <- redepth(dat$surface.area,ref="thin")
> m.all2 <- lmer(tcr~hrs+surface.area+(1|experiment),data=dat,REML=FALSE)
> summary(m.all2)
Linear mixed model fit by maximum likelihood  ['lmerMod']
```

```
Formula: tcr ~ hrs + surface.area + (1 | experiment)
   Data: dat

     AIC      BIC   logLik deviance df.resid
   481.5    493.7   -234.7    469.5       51

Scaled residuals:
    Min      1Q  Median      3Q     Max
-2.00841 -0.75982  0.06833  0.44372  2.73434

Random effects:
 Groups     Name        Variance Std.Dev.
 experiment (Intercept)   8.678   2.946
 Residual               214.491  14.646
Number of obs: 57, groups:  experiment, 3

Fixed effects:
                  Estimate Std. Error t value
(Intercept)        62.6867     4.3821  14.305
hrs                -3.5437     0.3424 -10.351
surface.areabeaker  33.5474     4.7516   7.060
surface.areathick   24.9105     4.7516   5.243

Correlation of Fixed Effects:
            (Intr) hrs    srfc.rb
hrs         -0.511
surfac.rbkr -0.542  0.000
surfc.rthck -0.542  0.000  0.500
>
> coefs <- data.frame(coef(summary(m.all2)))
> df.KR <- get_ddf_Lb(m.all2,fixef(m.all2))
> coefs$p.KR <- 2*(1-pt(abs(coefs$t.value),df.KR))
> coefs
                   Estimate Std..Error    t.value         p.KR
(Intercept)        62.686713  4.3821035  14.305165 3.445022e-10
hrs                -3.543669  0.3423596 -10.350723 2.965903e-08
surface.areabeaker 33.547368  4.7516317   7.060179 3.725350e-06
surface.areathick  24.910526  4.7516317   5.242520 9.713091e-05



>
```

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