Optimizing the Germination of Kidney Beans for Nutritional Improvement

by

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ABSTRACT

OPTIMIZING THE GERMINATION OF KIDNEY BEANS FOR NUTRITIONAL IMPROVEMENT

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Pulses are a nutritious source of fibre and protein, but they are also high in anti-nutritional factors such as alpha-galactosides, which cause flatulence in the gut. Among other methods, germination is a method that enhances nutritional value of pulses and is viable for scale-up. Therefore, the objective of this work was to optimize the germination process for a variety of bean, to reduce alpha-galactoside content. Six varieties of beans were analyzed for their sugar profile and proximate content, so that one could be chosen for germination. Response surface methodology was used to look at the relationship between the process variables time, temperature, and light, on the nutritional parameters of the germinated beans. Central composite faced design was used to generate twenty germinations, from which data was used to construct a theoretical model. Time and temperature were found to significantly affect the alpha-galactoside content of dark red kidney beans.

DEDICATION

Scavenging for their daily rice,

And wagging tongues on insignificant lies

Dejected in spirit, and toiling in vain suffering

Performing deeds that scathe fellow men

Aging with grey hair and then dying

Burdened with their own noxious bile

Like these foolish people (who live in vain)

Did you think that I too would fail?

-Bharathiyar

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AMG	Amyloglucosidase
ANOVA	Analysis of Variance
AOAC	Association of Official Agricultural Chemists
BF	Bean Flour
CCD	Central Composite Design
DM	Dry Matter
DPPH	2,2-diphenyl-1-picrylhydrazyl
DS	Digestible Starch
FC	Folin Ciocalteu's
FODMAP	Fermentable Oligo-, Di- and Monosaccharides and Polyols
GA	Gallic Acid
GBF	Germinated Bean Flour
GOPOD	Glucose Oxidase Peroxidase
GOS	Galactooligosaccharide
HPAEC	High Performance Anion Exchange Chromatography
IBS	Irritable Bowel Syndrome
IVPD	*In-vitro* Protein Digestibility
LAB	Lactic Acid Bacteria
LSD	Least Significant Difference
PAD	Pulsed Amperometric Detection
RS	Resistant Starch
RSM	Response Surface Methodology
TDF	Total Dietary Fiber
TE	Trolox Equivalent
TPC	Total Phenolic Content
TEPC	Total Extractable Phenolic Content

1 Introduction

The common bean (*Phaseolus vulgaris* L.) is a leafy plant that originated in the New World, but is now cultivated globally at latitudes from 52ºN to 32ºS and at elevations up to 3000 ft above sea level (Broughton et al. 2003; van Schoonhoven and Voysest 1991). The term 'bean' refers both to the immature pod and the mature dried seeds, however the focus of this thesis are the dried seeds. Dry beans come in a variety of market classes which all vary in size, shape and color (Kelly and Cichy 2012). The market classes that are preferred by Canadians are pinto, red kidney, and navy beans (Singh 2013). In 2017, India was the world's largest producer of dry beans at 6.3M tonnes, followed by Myanmar at 5.5M tonnes and Brazil at 3.0M tonnes (FAOSTAT 2017).

Beans are a good source of fibre, protein, starch, and vitamins, and are mainly consumed by middle- and low-class families (Ganesan and Xu 2017; Kutoš et al. 2003). The consumption of dry beans has been linked to a variety of health benefits, including the reduction of low density cholesterol, reduction of risk for heart disease and diabetes, as well as possessing anti-carcinogenic and anti-inflammatory properties (Brummer, Kaviani, and Tosh 2015; Ganesan and Xu 2017; Messina 2014; Suárez-Martínez et al. 2016). Despite their health benefits dry beans are often not consumed widely, because like many pulses, they can cause flatulence in consumers (Iriti and Varoni 2017; Minorsky 2003; Leterme and Carmenza Muñoz 2002; Schneider 2002).

The consumption of pulses causes flatulence due to the presence of alpha-galactosides, sugars composed of a sucrose backbone with galactose sugars attached to the glucose terminal (Martínez-Villaluenga, Frias, and Vidal-Valverde 2008). These galactosyls are attached by an alpha-1,6 bond, which is indigestible by enzymes in the human gut (Minorsky 2003). Instead, the gut microbiota digest these alpha-galactosides and produce both short chain fatty acids and gas. The production

of flatulence is especially severe for patients who suffer from gut-related diseases, such as Irritable Bowel Syndrome (IBS) and Crohn's disease, as the consumption of alpha-galactoside containing foods can worsen their symptoms (Erickson et al. 2017; Gibson and Shepherd 2005; Halmos et al. 2016; Nanayakkara et al. 2016). In order to encourage consumption of dry beans without ill side-effects, these alpha-galactosides should be removed from the beans.

There are several ways to process beans to remove alpha-galactosides, including extraction, thermal degradation, enzymatic degradation, and radiolytic degradation. Of the four methods, enzymatic degradation is the only one that achieves 100% removal of alpha-galactoside content from pulses. Methods of enzymatic degradation include germination, fermentation, and raw enzyme application, and all methods remove alpha-galactosides by using the enzyme alpha-galactosidase (natural or applied) to break down the sugars into their constituent products. Due to the activation of other enzymes, germination and fermentation can improve the nutritional content of pulses. While fermentation has been used industrially to transform pulses, such as soybeans, into ready-to-eat products like soy sauce and tempeh, germination of beans is still at a household level (Sozer, Holopainen-Mantila, and Poutanen 2017). A pilot plant germination machine was developed by Zamprogna et al. (2011), paving the way for industrial-scale germination of beans, and the development of germinated bean flour as a food ingredient. This machine was later used to germinate chickpeas and green peas and both pulses were found to have increased protein and free sugar contents (Erba et al. 2019).

Pulses are nutritious but cannot be eaten by patients with gut-related diseases. Hence, there lies an opportunity for research to be conducted in the field of transforming beans into value-added ingredients, to be used in diets and functional foods. Specifically, the controlled germination of beans can be optimized to ensure the maximum reduction of alpha-galactosides while preserving

or increasing the nutritional content. This thesis investigates the nutritional composition of different market classes of Canadian beans, then optimizes the germination process for the maximum nutritional value.

1.1 Research Objectives

The goal of this thesis is to evaluate the proximate composition and sugar profile of various market classes of Canadian beans and then optimize the germination process for the reduction of total alpha-galactosides, for a selected market class. The different market classes will be compared amongst each other according to nutritional value, sugar profile and alpha-galactoside content. From this investigation, a market class will be selected to be germinated under varying conditions, and both the nutritional value and sugar profile will be evaluated. Thus, the objectives of this thesis were the following:

1. Analyze the proximate composition and sugar profile of six selected market classes of Canadian beans.

2. Optimize the germination process on a selected market class of Canadian beans for the reduction of total alpha-galactosides.

The hypotheses for each of these objectives were the following:

1. Each bean would have a significantly different sugar profile.

2. The optimized process would be germination at 48hrs, 27.5℃, and 12 hours of light.

2 Literature Review

2.1 Acknowledgements

The following section is the modified manuscript that was published as follows:

Thirunathan, Praveena, and Annamalai Manickavasagan. "Processing methods for reducing alpha-galactosides in pulses." *Critical reviews in food science and nutrition* (2018): 1-12.

The review paper was written by Praveena Thirunathan and edited by Annamalai Manickavasagan, both from the School of Engineering, University of Guelph, Guelph, Ontario.

2.2 Abstract

Pulses are an excellent source of protein and dietary fibre and are consumed around the world. Their consumption has been recommended as part of a healthy diet. However, they contain various antinutrients such as tannins and trypsin inhibitors, as well as indigestible carbohydrates called alpha-galactosides. These oligosaccharides are fermented by the microorganisms in the gut, producing gas and causing flatulence in healthy individuals. While this flatulence is undesirable (and results in their low acceptance in the Western diet), alpha-galactosides have also been hypothesized to increase susceptibility to bowel diseases, and their presence in the gut worsens the symptoms of patients with irritable bowel syndrome. The elimination of alpha-galactosides by breeding is difficult as they play a vital role in maintaining seed viability through periods of drought and cold. There is a critical need to evaluate the various post-harvest processing methods, and their effect on alpha-galactoside removal to facilitate commercialization. This paper reviews the effectiveness of methods and processing conditions in alpha-galactoside removal from a variety of pulses.

2.3 Introduction

Pulses are the dried seeds obtained from members of the legume family. They are most well known for being an excellent source of protein (17-30% dry basis) and fiber (14-32 g of fiber per 100 g of pulses) (Boye, Zare, and Pletch 2010; Tosh and Yada 2010). Song et al. (2016) analyzed data from two US epidemiologic studies that spanned over thirty years, to examine the effect of animal and vegetable protein consumption on mortality rates. They found that increasing vegetable protein intake was positively associated with both the reduction of cardiovascular disease related mortality and the reduction of all-cause mortality in patients with at least one lifestyle risk factor (Song et al. 2016). Pulse consumption has been shown to improve satiety and metabolism of glucose and lipids, due to their high protein and fiber content, which makes their consumption ideal for preventing and managing obesity (McCrory et al. 2010; Rebello, Greenway, and Finley 2014). Abdullah et al. (2017) found that by following a low glycemic index, high fiber diet that included 100 grams of pulses per day, Canadians could generate a savings of CAD$6.2 million to CAD$62.4 million in healthcare costs of type 2 diabetes, and CAD$31.6 million to CAD$315.5 million in healthcare costs of cardiovascular diseases.

There are various anti-nutritional factors present in pulses, which reduce the availability of the nutrients already present in the pulse, thus reducing its dietary value (Sathe 2012). One of the main factors are trypsin inhibitors, compounds which inhibit the trypsin enzyme (Sathe 2012). This enzyme is vital for protein digestion, and thus the presence of trypsin inhibitors not only affects protein digestibility but may also reduce the already lacking sulfur amino acid content (Champ 2002). Tannins also impair protein digestibility by binding to the proteins and forming less digestible complexes, reducing bioavailability (Champ 2002).

Although not typically considered as an antinutritional factor in pulses, Fermentable Oligo-, Di- and Monosaccharides And Polyols (FODMAPs) are a group of short chain carbohydrates which cannot be digested by humans for energy (Gibson and Shepherd 2005; Halmos et al. 2014). They cause flatulence in the intestines by being fermented by the gut microbiota to produce short chain fatty acids, as well as hydrogen gas (Martínez-Villaluenga, Frias, and Vidal-Valverde 2008; Sosulski, Elkowicz, and Reichert 1982). This production of flatulence is a factor in the low acceptance of pulses into the Western diet (Minorsky 2003; Leterme and Carmenza Muñoz 2002; Schneider 2002). Gibson and Shepherd (2005) have postulated that these FODMAPs also play a negative role in the gut by increasing susceptibility to chronic bowel diseases, such as Crohn's disease and Irritable Bowel Syndrome (IBS), a disease which affects an average of 11% of the global population (Lovell and Ford 2012). Recent studies have implicated them as an irritant for patients who suffer from IBS (Erickson et al. 2017; Halmos et al. 2014; Nanayakkara et al. 2016; Staudacher and Whelan 2017).

There are five main categories of FODMAPs (Gibson and Shepherd 2005).

2.3.1 Polyols

Polyols are sugar alcohols (sorbitol, mannitol, xylitol and maltitol) that are widely prevalent in fruits such as apples, cherries, peaches and pears. They are used in the food industry as humectants and as sweeteners that also provide a cooling effect, suitable for products such as chewing gum (Gibson and Shepherd 2005).

2.3.2 Fructans

Fructans are short chains of fructose units with a glucose terminus, classified as either inulins or levans depending on the bonds between the fructose units (beta 1-2 or beta 2-6 respectively) (Gibson and Shepherd 2005). While fructans are found naturally in onions and wheat, their recognized pre-biotic effects make them a popular ingredient for various supplements (Al-Sheraji et al. 2013; Lee et al. 2012; Van den Ende, Peshev, and De Gara 2011).

2.3.3 Fructose

Fructose is generally considered a FODMAP when it is present in quantities that exceed glucose in a food. The transport mechanism used to absorb fructose is glucose dependent, but excess fructose leads to fructose malabsorption (Rumessen and Gudmand-Høyer 1988). Foods that contain an excess of fructose include apples and honey (Biesiekierski et al. 2011).

2.3.4 Lactose

Lactose is a common sugar found in dairy products, and it is digested in the gut using the enzyme lactase. However, different segments of the global population exhibit a lack of sufficient lactase activity in the gut, causing lactose intolerance and resulting in abdominal pain (Harrington and Mayberry 2008).

2.3.5 Galactooligosaccarides

Finally, the last category of FODMAPs is the galactooligosaccharides, so called because they are oligosaccharides that consist of sucrose with the addition of one or more galactosyl residues (Martínez-Villaluenga, Frias, and Vidal-Valverde 2008). They are prevalent in vegetables like

Brussels sprouts and cabbage, and pulses such as beans, lentils, and peas (Biesiekierski et al. 2011; Gibson and Shepherd 2005). Of these galactooligosaccharides, there exists a subcategory called alpha-galactosides, comprised of sugars that have an alpha-1,6-galactosyl residue attached at the end.

Biesiekierski et al. (2011) developed FODMAP profiles for various processed foods and found that the most prevalent category of FODMAPs in boiled or canned pulses were total alpha-galactosides (denoted as GOS (GalactoOligoSaccharides) in their study). Thus, to reduce the production of flatulence from pulse consumption, these alpha-galactosides must be removed from the pulse.

Scientists have attempted to breed lines of pulses with reduced alpha-galactoside content and were moderately successful (Clarke and Wiseman 2000; de Lumen 1992; Frias et al. 1999; Jones et al. 1999). Kerr (1992) patented a DNA sequence that inhibited galactol synthase activity, which would then reduce alpha-galactoside levels in the seed. Despite these efforts, the problem with eliminating alpha-galactosides at the pre-harvest stage is they are implicated in various roles in protecting the seed, including cold acclimation (Gilmour et al. 2000), drought resistance (Obendorf 1997), seed stability during storage (Bernal-Lugo and Leopold 1995) and their role in being a starter material for germination (Blöchl, Peterbauer, and Richter 2007). Because of the importance of alpha-galactosides in preserving seed viability, processing treatments at the post-harvest stage are a more promising alternative to reduce the alpha-galactoside content in pulses. In order to increase pulse consumption, a critical evaluation of the various processing methods to remove alpha-galactosides, and the factors that affect removal, is required in order to successfully develop and commercialize a processing method, to be used at the post-harvest stage. This review

characterizes and compares the various processing methods used to remove alpha-galactosides from pulses.

2.4 Alpha-galactosides in pulses

The synthesis of alpha-galactoside starts with the enzyme galactinol synthase, which forms galactinol out of myo-inositol and galactose. Sucrose then accepts the galactinol and raffinose synthase catalyzes the reaction to form raffinose and myo-inositol (Martínez-Villaluenga, Frias, and Vidal-Valverde 2008). Stachyose and verbascose are formed in the same manner using raffinose (for stachyose) or stachyose (for verbascose), and galactinol as the substrates, and stachyose synthase as the enzyme (Martínez-Villaluenga, Frias, and Vidal-Valverde 2008). In lentils, the pathway is slightly altered in that when verbascose production is blocked, ciceritol content increases instead of stachyose content (Jones et al. 1999).

The most prevalent alpha-galactoside differs from pulse to pulse (Table 2.1). Stachyose was found to be the prevalent alpha-galactoside in *Lens culinaris*, *Glycine max* and *Pisum sativum* (Tahir, Vandenberg, and Chibbar 2011; Wang, Hatcher, and Gawalko 2008). Verbascose content varies widely between pulses, from near absent in *Cicer arietinum* to 7.6% (DM) in *Cajanus cajan* (Devindra et al. 2011; Wang et al. 2010). Ciceritol is a cyclic alpha-galactoside that constitutes more than 50% of the total alpha-galactoside content in *Cicer arietinum* (Xiaoli et al. 2008). It is also found in *Lens culinaris*, although not in large quantities (Faris, Takruri, and Issa 2013). Ajugose is a higher order alpha-galactoside that has been successfully isolated from *Vigna mungo* (Kotiguda, Peterbauer, and Mulimani 2006). Raffinose tends to be the sugar that is the least prevalent in the seed, due to the nature of the alpha-galactoside synthesis pathway.

2.5 Removal methods

The following sections focus on the various methods used to remove alpha-galactosides from pulses.

2.5.1 Extraction

Alpha-galactoside extraction is based on the principle that the sugars are both water and alcohol soluble (Dey 1980). The pulses can have the solvent passed through their material, either passively (as in soaking) or actively (by using heat to speed up extraction). This allows for the solvent to carry off the alpha-galactoside from the pulse seed, and the extract can either be discarded, or kept for purification of the alpha-galactoside (Giannoccaro, Wang, and Chen 2006).

2.5.1.1 Soaking

Soaking is the oldest and most traditional method of removing alpha-galactosides from pulses. The dried pulses are placed in a pot of water and allowed to rehydrate over a long period of time (generally overnight). The water-soluble alpha-galactosides are drawn out by osmosis into the soaking water. The addition of water also initiates a metabolic process in the pulse, which serves to further degrade the alpha-galactoside (Vidal-Valverde, Frías, and Verde 1992). By summing the content of alpha-galactosides in the soaking water and soaked lentil (*Lens culinaris*), and comparing it to the content of alpha-galactosides in the raw lentil, they found that leaching only accounted for a small portion of the decrease in alpha-galactoside content (Vidal-Valverde, Frías, and Verde 1992). The theory of a metabolic process was further explained by the increase in fructose content and the appearance of glucose content in the soaked lentil, while glucose was non-

Table 2.1 Alpha-galactoside Content in Pulses (% Dry Basis)

Pulse Type	Raffinose	Stachyose	Verbascose	Ciceritol	Ajugose	Reference
Lentils *Lens culinaris*	0.10-0.53	1.10-2.97	ND-0.97	0.24-1.01	–	(Frias et al. 1994; Vidal-Valverde et al. 1993)
Green Peas *Pisum sativum*	0.83-1.12	2.48-3.69	1.07-2.22	–	–	(El-Adawy et al. 2003; Wang, Hatcher, and Gawalko 2008)
Common Beans *Phaseolus vulgaris*	0.13-0.71	3.04-3.77	0.023-1.51	–	–	(Silva and Braga 1982; Ning Wang et al. 2010)
Chickpeas *Cicer arietinum*	0.52-0.72	1.64-3.06	ND-0.70	3.04-5.06	–	(Wang et al. 2010; Xiaoli et al. 2008)
Mung Beans *Phaseolus aureus*	0.30-0.43	0.63-1.52	2.40	–	–	(El-Adawy et al. 2003; Fan, Zang, and Xing 2015; Su and Chang 1995)
Black Gram *Vigna mungo*	trace-0.76	0.25-0.89	1.12-3.44	–	0.81-1.68	(Girigowda, Prashanth, and Mulimani 2005; Reddy and Salunkhe 1980)
Cowpeas *Vigna unguiculata*	0.52-2.50	2.04-4.20	–	–	–	(Ibrahim et al. 2002; Somiari and Balogh 1993)
Pigeon peas *Cajanus cajan*	0.52-1.22	0.72-1.82	3.60-7.60	–	–	(Devindra et al. 2011; Mulimani and Devendra 1998)
Soybeans *Glycine max*	0.59-3.19	4.10-14.10	0.15-0.66	–	–	(Fan, Zang, and Xing 2015; Kaczmarska et al. 2017)

– indicates the sugar is not present in the pulse. ND indicates the sugar was not detected.

detectable in the raw lentil. Thus, unlike the other processing methods, soaking utilizes two mechanisms of alpha-galactoside removal, extraction and enzymatic degradation.

A brief overview of various studies that utilized soaking as a method of removal is shown in Table 2.2. From there, we see that different pulses show different levels in alpha-galactoside reduction, despite being treated with the same conditions. In particular, brown pigeon peas exhibit alpha-galactoside losses of 20-40%, while cream pigeon peas exhibit losses of 50-90% (Table 2.2). This is a reflection of the importance of the seed matrix and seed coat when it comes to alpha-galactosides diffusing out of the pulse (Upadhyay and Garcia 1988). Another finding is that soaking pulses in a sodium bicarbonate solution drastically increases the amount of alpha-galactosides that are removed from the pulse. This could be attributed to the change in permeability of the seed coat in a high-pH solution, facilitating the diffusion of alpha-galactosides through the seed (Vijayakumari, Pugalenthi, and Vadivel 2007). Finally, longer amounts of soaking time remove a greater amount of alpha-galactosides (Table 2.2).

Water temperature is one of the predominant factors affecting the reduction of alpha-galactosides in soaked pulses. At high temperatures, the seed coat of the pulse is rendered more permeable, allowing more alpha-galactosides to leach out. In addition to this, alpha-galactosidase activity within the seed increases, allowing for more sugar degradation. Dey, Campillo, and Lezica (1983), found that the alpha-galactosidase isolated from *Lens culinaris* had an optimal activity between 20-50°C while Baldini, Draetta and Park (1985) found that alpha-galactosidase isolated from *Phaseolus vulgaris* had an optimum activity at 55°C. However, there are limits to increasing the enzymatic activity through rising temperature, as illustrated by Coffigniez et al. (2018). They constructed a model to examine the reaction and diffusion mechanisms present in soaking *Vigna unguiculata* at different temperatures and found that at lower temperatures (30°C), enzymatic

Table 2.2 Changes in Alpha-Galactoside Content in Pulses during Soaking.

	Soaking Parameters				Change in Alpha-Galactoside Content								
Pulse Name	B:W[@] ratio (w/v)	Time (hr)	Temp.[#] (°C)	Type of water	Raffinose		Stachyose		Verbascose		Ciceritol		Reference
					Init.[$]	Final	Init.[$]	Final	Init.[$]	Final	Init.[$]	Final	
Soybeans	1:10	12	Room temp.	Milli Q	0.984 ±0.008*	0.722 ±0.006*	1.646 ±0.010*	1.315 ±0.008*	N/A		N/A		(Singh and Kayastha 2013)
Chickpeas	1:10	16	20	Tap water	0.320 ±0.030*	0.260 ±0.020*	1.770 ±0.060*	1.250 ±0.130*	N/A		2.760 ±0.100*	2.030 ±0.090 *	(Aguilera et al. 2009)
Common Bean (Pink-mottled cream)	1:10	16	20	Tap water	0.160 ±0.020*	0.130 ±0.010*	2.530 ±0.010*	1.850 ±0.100*	N/A		N/A		
Common Bean (White)	1:10	16	20	Tap water	0.190 ±0.080*	0.120 ±0.040*	2.480 ±0.100*	1.830 ±0.070*	N/A		N/A		
Bauhinia purpurea	1:10	6	Not indicated	Distilled water	0.540	0.460	1.170	1.050	0.950	0.770	N/A		(Vijayakumari, Pugalenthi, and Vadivel 2007)
	1:10	6	Not indicated	Water with 0.02% $NaHCO_3$	0.540	0.440	1.170	0.990	0.950	0.700	N/A		
Pigeon Peas	1:10	16	35	Water with 1% $NaHCO_3$	1.420 ±0.030	0.420 ±0.020	1.650 ±0.040	0.580 ±0.060	4.950 ±0.280	2.300 ±0.000	N/A		(Devindra and Aruna 2017)
	1:10	16	35	Water with 2% $NaHCO_3$	1.420 ±0.030	0.370 ±0.040	1.650 ±0.040	0.520 ±0.010	4.950 ±0.280	2.400 ±0.020	N/A		
Soybeans	1:3	12-14	Not indicated	Tap water	1.220 ±0.020	0.910 ±0.030	3.410 ±0.080	2.720 ±0.040	N/A		N/A		(Egounlety and Aworh 2003)
Cowpeas	1:3	12-14	Not indicated	Tap water	0.780 ±0.060	0.590 ±0.040	3.530 ±0.100	2.940 ±0.080	N/A		N/A		
Groundbean	1:3	12-14	Not indicated	Tap water	0.980 ±0.040	0.680 ±0.020	2.750 ±0.070	1.990 ±0.060	N/A		N/A		
Pigeon peas (Brown)	1:3	9	Not indicated	Distilled water	0.423 ±0.057	0.268 ±0.008	0.857 ±0.065	0.670 ±0.008	1.067 ±0.110	0.820 ±0.010	N/A		(Oboh et al. 2000)
Pigeon peas (Cream)	1:3	9	Not indicated	Distilled water	0.620 ±0.003	0.033 ±0.004	1.335 ±0.006	0.632 ±0.032	1.562 ±0.070	0.824 ±0.040	N/A		
Black Gram	1:10	8	37	Not indicated	0.360 ±0.000	0.290 ±0.020	0.420 ±0.010	0.270 ±0.010	3.160 ±0.020	2.950 ±0.020	N/A		(Girigowda, Prashanth, and Mulimani 2005)
	1:10	12	37	Not indicated	0.360 ±0.000	0.250 ±0.000	0.420 ±0.010	0.250 ±0.000	3.160 ±0.020	2.580 ±0.010	N/A		
	1:10	16	37	Not indicated	0.360 ±0.000	0.210 ±0.000	0.420 ±0.010	0.220 ±0.010	3.160 ±0.020	2.260 ±0.010	N/A		

N/A indicates that this sugar was not tested for. Values are reported as mean ± standard deviation. Labels in parentheses represent the variety of pulse. @ = Bean:Water. w/v = weight to volume. # = Temperature. $ = Initial value. * = indicates value was converted from g/kg dry matter to % dry matter.

degradation prevailed in reducing alpha-galactoside content. However, at higher temperatures (60/95°C), the enzymes would be deactivated, and thus diffusion of alpha-galactosides into the water would prevail (Coffigniez et al. 2018).

Time is also a factor in soaking extraction efficiency. By leaving the pulses in the aqueous solvent for a longer period of time, further diffusion and metabolism of the alpha-galactosides is enabled. Upadhyay and Garcia (1988) examined the effect of soaking on alpha-galactoside content of *Vigna unguiculata* and found that soaking for 18 hours resulted in a significant ($p < 0.05$) decrease in only stachyose content, and not raffinose or verbascose, both of which are only present in small amounts. Vijayakumari, Siddhuraju, and Janardhanan (1996) reported a significant ($p < 0.05$) decrease in raffinose, stachyose and verbascose in Kerala *Mucuna monosperma* when they were soaked for 12 hours and above, while soaking only significantly decreased stachyose and verbascose in Tamil Nadu *Mucuna monosperma*, but not raffinose, at soaking for 12 hours and above. However, for the wild pulse *Bauhinia purpurea L.*, significant ($p < 0.05$) decreases were only found in verbascose after 6 hours, while raffinose and stachyose didn't decrease significantly (Vijayakumari, Pugalenthi, and Vadivel 2007).

Additionally, the greatest decrease in alpha-galactosides generally occurs in the beginning of soaking, as evidenced by Somiari and Balogh (1993). When soaking *Vigna unguiculata* flour, they found that the greatest decrease in raffinose and stachyose occurred after 4 hours, with a decrease of 12% in mean raffinose content and 19% in mean stachyose content (Somiari and Balogh 1993). Further soaking only reduced raffinose and stachyose content by an additional 5-8% every additional 4 hours (Somiari and Balogh 1993). This trend was also observed by Mulimani and

Devendra (1998) when soaking *Cajanus cajan* flour, where they found that the greatest decrease in raffinose (19%) occurred at 8 hours, and at 4 hours for stachyose (34%) and verbascose (31%).

In general, the longer the pulses are soaked, the greater the decrease is in the oligosaccharide content, however the time it takes to obtain a significant ($p < 0.05$) decrease is relevant on the type of pulse. These differences in extraction are due to the diffusion rate between the various sugars, as well as their solubility (Upadhyay and Garcia 1988). The diffusion rate of the sugars depends on each pulses' seed coat thickness and permeability, while the solubility is a trait inherent for each sugar (Upadhyay and Garcia 1988).

Since soaking is a common household processing method, studies have attempted to optimize the factors important for soaking in order to maximize the reduction in alpha-galactosides. A study by Rakshit et al. (2015) was conducted where soaking time, water temperature, water pH and bean:water ratio were varied in a response surface model experiment to determine the optimal soaking conditions for *Vigna mungo*. The resulting model showed that only pH significantly ($p < 0.05$) affected the alpha-galactoside content in *Vigna mungo*, the term having a negative coefficient indicating that a rise in water pH results in a lower total alpha-galactoside content (Rakshit et al. 2015). The interaction effect between temperature and time was also significant in this model. Many studies have looked at soaking pulses for long periods of time (9-16 hrs) (Aguilera et al. 2009; Devindra and Aruna 2017; Egounlety and Aworh 2003; Oboh et al. 2000; Singh and Kayastha 2013), thus Vidal-Valverde et al. (2002b) looked at how water temperature, bean to water ratio, light exposure and retention of soaking water affected short time (1 hour) soaking procedures for *Phaseolus vulgaris*. They found that by raising the temperature, performing the method in the absence of light, and by removing the soaking water afterwards, the total alpha-galactoside content was minimized in the whole seed and in the flour (Vidal-Valverde et al. 2002b)

2.5.1.2 Cooking

Cooking in this context refers to the process of boiling a food in water at 99°C. Although this process involves applying heat to the food, it is not classified as thermal degradation, as the temperature is insufficient to degrade the alpha-galactosides within the pulses. However, aside from the usual mechanism of diffusion, it has been postulated that the additional heat enables the heat hydrolysis of alpha-galactosides within the pulse (Onigbinde and Akinyele 1983). Unlike soaking, enzymatic reaction would not be a predominant mechanism in this method of processing, as the high heat would inactivate the alpha-galactosidase enzyme. Generally, studies indicate a trend of increased alpha-galactoside removal with increased cooking time. Wang et al. (2003) found that the greatest decrease of raffinose, stachyose and verbascose in *Pisum sativum* occurred at 30 min (mean values of 39.2%, 48.1% and 40.4% respectively). Similar trends were found with cooking *Vigna unguiculata* flour (Somiari and Balogh 1993) and *Cajanus cajan* flour (Mulimani and Devendra 1998).

One concerning side effect of cooking is that when pulses are cooked without a presoaking treatment, the levels of alpha-galactosides increase. Stachyose content was found to increase in unsoaked cooked *Cicer arietnum* by 133% and in unsoaked cooked *Glycine max* by 57%, while verbascose content increased in unsoaked cooked *Pisum sativum* by 282% (Han and Baik 2006). Rao and Belavady (1978) also found similar trends when cooking unsoaked *Cajanus cajan*, *Cicer arietinum*, *Vigna mungo*, and *Phaseolus aureus*: all samples exhibited an increased raffinose and verbascose+stachyose (the sugars were not analyzed separately) content after processing. This phenomenon can be attributed to some alpha-galactoside content being bound to proteins within the seed. As the raw seed, these bound oligosaccharides are not quantified, but when these proteins

are denatured by the cooking process, the alpha-galactosides are released and become free within the seed (Rao and Belavady 1978).

2.5.2 Thermal degradation

Thermal degradation uses high amounts of heat to hydrolyze the alpha-galactosides within the pulses, to the detriment of the other nutritional compounds in the pulses. Raffinose was found to degrade at 210°C into two compounds: fructose, and a proposed alpha-galactose (1-6) glucose dimer (melibiose) (Forgo et al. 2013). The heat can also affect antinutritional factors such as trypsin, but through this method, the alpha-galactosides are unrecoverable for later uses (Rehman and Shah 2005).

2.5.2.1 Autoclaving

Meant to simulate traditional methods of home cooking pulses, autoclaving applies both heat and pressure to pulses so that the alpha-galactosides are removed. Mubarak (2005) found that both autoclaving and cooking *Phaseolus aureus* seeds produced roughly the same decrease in raffinose and stachyose. However, autoclaving was found to remove 49.2- 56.0% of total alpha-galactosides from unsoaked *Vicia faba*, *Vigna unguiculata*, *Lens culinaris* and *Phaseolus vulgaris*, in comparison to traditional cooking which only removed 41.5- 47.6% of total alpha-galactosides (Abdel-Gawad 1993). This trend was also found in the water-soaked pulses, where autoclaving removed 56.8-70.0% of total alpha-galactosides, compared to traditional cooking which removed 49.7-64.0% (Abdel-Gawad 1993). It is important to note that the cooking process took a longer time (60 min) than the autoclaving process (20 min). Since the addition of a presoaking treatment was shown to remove more alpha-galactosides, Siddhuraju and Becker (2001) examined the effect of various additions to the presoaking water on the removal of alpha-galactosides from autoclaved

Mucuna pruriens var *utilis*. They found that while all presoaked autoclaved seeds had a larger decrease in alpha-galactosides than autoclaved raw seeds, the tamarind pulp soaked and alkaline soaked seeds exhibited a greater decline (68-71%) than citric acid soaked (63%) or water soaked (62%) seeds (Siddhuraju and Becker 2001). Vijayakumari, Siddhuraju, and Janardhanan (1996) noted that when autoclaving *Mucuna monosperma* seeds for varying amounts of time, the decrease in alpha-galactosides increased with increasing time. Silva and Braga (1982) investigated the effects of bean to water ratio on the removal of alpha-galactosides from autoclaving *Phaseolus vulgaris* and found that a bean to water ratio of 1:10 w/v facilitated a greater removal of raffinose (73%) and stachyose (48%) than a ratio of 1:3 w/v (41% and 18% respectively). The pressure induced by the autoclave forces water into the seed, allowing for a more efficient extraction of alpha-galactosides than mere cooking (Malki and Waisel 1987).

2.5.2.2 Extrusion

Extrusion is a process which applies shear forces and pressure in addition to thermal stresses in order to transform the feed (in this case, a paste made of pulses) into a novel product with transformed functional and nutritional properties. Various studies have shown mixed effects in the efficiency of alpha-galactoside reduction. Ai et al. (2017) used extrusion on four variety classes of *Phaseolus vulgaris*, with the final barrel temperature set to either 120°C or 140C°C and found that while raffinose content significantly decreased by 58-82%, stachyose content actually increased by 38-121%. Another study used a final barrel temperature of 160°C on the extrusion of various pulses (*Cicer arietinum*, *Lens culinaris* and *Pisum sativum*), and found insignificant results on the raffinose, stachyose and ciceritol content in the extruded raw pulse flours, whether it was an increase or decrease (Berrios et al. 2010). However, the specially formulated pulse flours (where starch, fiber and flavorings were added) showed a significant decrease in oligosaccharide content

(Berrios et al. 2010). Berrios et al. (2002) found the pH of the extrusion feed had no effect on the raffinose and stachyose content of beans extrudate. While extrusion at low temperatures (85°C) would be preferred to preserve the nutritional content of pulses, Kelkar et al. (2012) found that it only reduced raffinose content significantly, and not stachyose content. Frias et al. (2011) had more success with extrusion of *Pisum sativum* at moderate temperatures (129°C, 135°C and 142°C) with a significant reduction of 28% in total alpha-galactosides (raffinose, stachyose and verbascose) at 142°C.

2.5.3 Enzymatic degradation

The enzyme alpha-galactosidase is naturally present in pulses and can be utilized by different methods to break down the alpha-galactosides. Alpha-galactosidase cleaves the galactosyls at the alpha-1,6 linkage, producing sucrose molecules and free galactose residues (De Andrade Taborda, Da Costa Cardoso, and Karp 2016). The processing method can utilize the enzyme already present in the pulse (such as in sprouting or fermentation), or the enzyme can be added separately to the whole pulse or the pulse flour. Sprouting (or germination) utilizes the dormant alpha-galactosidase within the seed to degrade the alpha-galactosides as the seed sprouts. Fermentation uses alpha-galactosidase from bacteria and yeast, instead of the seed, to degrade the alpha-galactosides. Finally, enzyme treatment is a method where the alpha-galactosidase enzyme is externally synthesized and added to the seed.

2.5.3.1 Enzyme treatment

This method uses alpha-galactosidase applied directly to the whole pulse or pulse flour, which is then incubated to increase enzyme activity. By targeting the alpha-galactosides directly, both the

nutritional and antinutritional factors are left untouched, and alpha-galactosides can be degraded completely.

In comparison to the other methods, enzyme treatment has shown to be excellent at removing alpha-galactosides from a variety of pulses. Devindra and Aruna (2017) purified alpha-galactosidase from *Aspergillus niger* and the semi purified extract was used to treat *Cajanus cajan* flour, resulting in undetectable levels of raffinose, stachyose and verbascose. These results are in agreement with Mulimani and Devendra (1998) who also reported complete removal of alpha-galactosides from *Cajanus cajan* flour. Both papers noted that enzyme treatment was more effective than soaking or cooking methods. Somiari and Balogh (1993) reported an 93% reduction in raffinose and 82% reduction in stachyose when *Vigna unguiculata* flour is incubated with alpha-galactosidase at 50°C for 2 hours, while Anisha and Prema (2008) reported a decrease in raffinose and stachyose by 97.5% and 93.2% (respectively) in *Dolichos biflorus* and by 96.3% and 91.8% in *Phaseolus aureus*.

Different factors such as enzyme source, incubation time and temperature, and pulse powder particle size all play a role in the efficacy of enzymatic treatment. When Matella et al. (2005) looked at enzyme treatment compared to soaking or germination for *Phaseolus vulgaris*, enzyme treatment only removed 30% to 51% of oligosaccharides, compared to germination which removed 60% to 70% of oligosaccharides. However, this study used a lower incubation temperature (23°C) and a lower incubation time (1 hour) than typical enzymatic treatment studies that used an incubation temperature range of 37-55°C and a time range of 2-4 hours (Anisha and Prema 2008; Devindra and Aruna 2017; Mulimani, Thippeswamy, and Ramalingam 1997; Mulimani and Devendra 1998; Somiari and Balogh 1993). Mansour and Khalil (1998) investigated the influence of alpha-galactosidases purified from various bacteria, and incubated *Cicer arietinum* flour for

differing incubation times and at different enzyme concentrations. They found that increasing the incubation time and the amount of enzyme reduced more raffinose and stachyose in the flour sample. Although Somiari and Balogh (1995) showed us that a smaller particle size enhanced removal of alpha-galactosides from *Vigna unguiculata* flour, Song, Chang and Ibrahim (2009) found that a median particle size (60 mesh) had the greatest reduction of alpha-galactosides in *Phaseolus vulgaris* var. *Pinto* (pinto bean) flour. They also found that pre-steaming pinto bean flour at 115°C enabled a greater reduction in alpha-galactosides than pre-steaming at 120°C (Song, Chang, and Ibrahim 2009)

One of the drawbacks of this method is the sensory quality of the pulse is affected by the application of crude enzyme. Song, Chang and Ibrahim (2009) found that enzyme treated pinto bean paste had a significantly higher astringency, raw beany off-flavor, and bitterness score than untreated pinto bean paste, as the crude alpha-galactosidase enzyme preparation contained other enzymes which hydrolyzed various compounds in the bean paste, leading to the production of off flavors.

2.5.3.2 Germination

Germination is a method in which plant seeds are deliberately placed in optimal environmental conditions, such that the seed is able to sprout the germ within. The act of developing the germ into a sprout uses up the sugars and also modifies the starch, lipid and protein content within the seed (Matella et al. 2005). It has been widely used as a method to improve the nutritional content of various seeds such as wheat, sesame, and oats (Chavan, Kadam, and Beuchat 1989; Hahm, Park, and Martin Lo 2009). The germination process does not affect the sensory qualities of the resulting seed flour, which makes it an attractive option for the inclusion of various grains and legumes into

a diet (Torres et al. 2007; Uwaegbute, Iroegbu, and Eke 2000). As the seed develops its sprout, alpha-galactosidase activity is enhanced, allowing for it to break down the alpha-galactoside content in the seed. When Petrova, Marinova, and Tchorbanov (2010) examined the alpha-galactosidase activity in sprouting *Lens culinaris* seeds, they found that activity peaked on the 3rd day of germination, slowly decreasing afterwards. In addition to this, alpha-galactosides have been shown to be critical in the early germination of *Pisum sativum* seeds. By blocking alpha-galactosidase activity within the germinating seed, germination was delayed for several days (Blöchl, Peterbauer, and Richter 2007). This process is naturally only applicable to the whole pulse, but the sprouted pulse can be ground into flour afterwards, with varying functionalities and nutritional properties.

Generally, seeds are first presoaked to take up moisture before allowing them to germinate under controlled conditions. This presoaking may have a synergistic effect on the reduction of alpha-galactoside content, not only diffusing the sugars out into the soaking medium, but also activating the alpha-galactosidase within the seed. Light exposure plays a curious part in the germination of pulses, and its presence or absence has different effects. Vidal-Valverde et al. (2002a) examined the effects of light exposure compared to germinating *Phaseolus vulgaris* in darkness. They found that germinating *Phaseolus vulgaris* in the dark after 6 days removed total alpha-galactosides (raffinose and stachyose) to a non-detectable level, compared to germinating in the light which reduced total alpha-galactosides by 98% (Vidal-Valverde et al. 2002a). However, with *Lens culinaris*, both germination in darkness and in light reduced total alpha-galactosides (raffinose, stachyose and ciceritol) to nondetectable levels. *Pisum sativum* showed a near equal total alpha-galactosides (raffinose, stachyose, and verbascose) content when germinated in light (98.1%) compared to germination in the dark (97.7%) (Vidal-Valverde et al. 2002a).

Table 2.3 shows the variations in germination conditions and their effect on alpha-galactoside reduction. When we look at pulses germinated in darkness compared to pulses germinated in light, we find that for shorter periods of times, darkness is able to completely remove stachyose and verbascose, while 8 hours of light is able to completely remove raffinose and verbascose. However, when pulses are left for longer periods of time, both complete light exposure and complete darkness are able to remove 80-100% of alpha-galactosides. Soaking time is also positively correlated with alpha-galactoside removal, which is logical given that soaking is another effective method to remove alpha-galactosides. Temperature of germination does not play a significant role, however there have not yet been studies where pulses are germinated at extremely high (>30°C) or extremely low (<20°C) temperatures yet. It can be theorized that enzyme activity is increased at higher temperatures, and thus higher temperatures would facilitate a more complete removal of alpha-galactosides from the pulse.

2.5.3.3 Fermentation

Pulse fermentation occurs by either utilizing the microorganisms already present within the seed (Natural fermentation), or by inoculating the seeds with various strains of bacteria or molds (Induced fermentation). During fermentation, the microorganisms produce enzymes, which consume both nutritional and antinutritional factors. In this case, the alpha-galactosides are consumed by alpha-galactosidase present in the microbiota. The pulse emerges as a changed product, with novel functional and nutritional properties.

The microorganisms involved in the natural fermentation process are varied and differ according to the pulse. When *Phaseolus vulgaris* is naturally fermented, the microorganisms responsible for the process consist of 62% coliforms and 36% *Lactobacillus* strains, with minimal amounts of

Table 2.3 Changes in Alpha-Galactoside Content in Pulses during Germination.

	Germination Conditions				Change in Alpha-Galactoside Content								
Pulse Name	Light	Time (hr)	Temperature (°C)	Presoak Time (hr)	Raffinose		Stachyose		Verbascose		Ciceritol		Reference
					Init.$	Final.	Init.$	Final.	Init.$	Final	Init.$	Final	
Mung Beans	Darkness	72	28	0	0.300	trace	1.500	trace	2.700	trace	N/A		(Åman 1979)
Chickpeas	Darkness	72	28	0	1.000	0.300	2.800	0.700	trace	ND	N/A		
Mung Beans	Darkness	48	25-27	4	2.600	2.660	2.830	ND	3.460	ND	N/A		(Jaya and Venkataraman 1981)
	Darkness	96	25-27	4	2.600	1.210	2.830	ND	3.460	ND	N/A		
Chickpeas	Darkness	48	25-27	4	1.440	0.780	2.580	1.080	3.800	ND	N/A		
	Darkness	96	25-27	4	1.440	1.020	2.580	ND	3.800	ND	N/A		
Pigeon peas (Brown)	8hr Daylight	72	25	9	0.423 ±0.057	ND	0.857 ±0.065	0.485 ±0.049	1.067 ±0.110	0.120 ±0.005	N/A		(Oboh et al. 2000)
	8hr Daylight	96	25	9	0.423 ±0.057	ND	0.857 ±0.065	0.473 ±0.023	1.067 ±0.110	ND	N/A		
Pigeon peas (Cream)	8hr Daylight	72	25	9	0.620 ±0.003	ND	1.335 ±0.006	0.371 ±0.002	1.562 ±0.070	ND	N/A		
	8hr Daylight	96	25	9	0.620 ±0.003	ND	1.335 ±0.006	0.597 ±0.015	1.562 ±0.070	ND	N/A		
Common Beans	24hr Light	144	20	6	0.110 ±0.010	0.010 ±0.000	0.500 ±0.010	ND	ND		ND		(Vidal-Valverde et al. 2002a)
	Darkness	144	20	6	0.110 ±0.010	ND	0.500 ±0.010	ND	ND		ND		
Lentils	24hr Light	144	20	6	0.380 ±0.010	ND	1.770 ±0.080	ND	ND		0.620 ±0.040	ND	
	Darkness	144	20	6	0.380 ±0.010	ND	1.770 ±0.080	ND	ND		0.620 ±0.040	ND	
Green Peas	24hr Light	144	20	6	0.560 ±0.030	0.100 ±0.010	2.240 ±0.060	ND	2.390 ±0.010	ND	ND		
	Darkness	144	20	6	0.560 ±0.030	0.120 ±0.000	2.240 ±0.060	ND	2.390 ±0.010	ND	ND		

N/A indicates that this sugar was not tested for. Labels in parentheses represent the variety of pulse. $ = Initial value. ND = no detection of this carbohydrate.

Streptococcus strains. The *Lactobacillus* strains were found to be *Lactobacillus casei* and *Lactobacillus plantarum*, indicating that the fermentation was performed with these bacteria (besides the coliforms) (Granito and Álvarez 2006). This is consistent with many foods developed by natural fermentation, where it has been found that the major bacteria responsible for the fermentation process are Lactic Acid Bacteria (LAB), microorganisms which produce lactic acid as a byproduct of their metabolism (Blandino et al. 2003) The production of lactic acid lowers the pH extensively, allowing for the exclusion of other microorganisms that may negatively affect the fermentation process.

One of the foremost factors in successful fermentation is the duration for which the pulse is fermented. By letting the pulse ferment for different periods of time, the levels of various nutrients change due to the slowing of enzymatic activity. Egounlety and Aworh (2003) found that by fermenting *Glycine max* with the mold *Rhizopus oligosporus* over several days, the stachyose content decreased from 1.25% at 0 hr to 1.1% at 24 hr, then to 0.6% at 48 hr. A similar decreasing trend was found in stachyose contents for *Vigna unguiculata* and *Macrotyloma geocarpa* (Egounlety and Aworh 2003).

Several studies have considered the effects of allowing the pulse to ferment "naturally" (i.e., with the microbiota found in the seed) as compared to inoculating the pulse with a specific microorganism or a combination of microorganisms to optimize the resulting nutritional content. Kaczmarska et al. (2017) compared naturally fermented *Lupinus angustifolius* (lupin) and *Glycine max* (soybean) with fermentation using a yogurt culture, in which contained the bacteria *Lactobacillus delbrueckii* subsp. *bulgaricu*s and *Streptococcus thermophillus*. The results found that although both induced and natural fermentation increased raffinose content in lupin seed flour

(induced by 151%, natural by 279%), and increased verbascose content in soybean flour (induced by 38%, natural by 79%), induced fermentation reduced more total alpha-galactosides (raffinose, stachyose and verbascose) than natural fermentation in both lupin (induced by 22%, natural increased by 16%) and soybean (induced by 44%, natural by 18%) (Kaczmarska et al. 2017). Induced fermentation of *Vigna unguiculata* with *Lactobacillus plantarum* after 48 hr also showed a decrease in total alpha-galactosides (raffinose, stachyose and verbascose) by 92%, compared to natural fermentation, which decreased total alpha-galactosides by 85% (Azeke et al. 2005).

A general comparison of several studies has been provided in Table 2.4. Compared to induced fermentation by introducing various bacterium, natural fermentation seemed to provide the highest reduction in alpha-galactosides when fermenting various pulses. While it would be expected that fermenting pulse flour as compared to the whole bean would result in a greater reduction of alpha-galactosides, this was not the case with Granito et al. (2002). However, this factor would require further study before a conclusive judgement can be made. Time and temperature both play a role, as longer fermentation periods and higher temperatures enable a larger reduction of alpha-galactosides from pulses.

2.5.4 Radiolytic degradation

By applying gamma radiation, the alpha-galactosides are degraded through the cleavage of the alpha-1,6 bond between the galactosyls due to the radical electrons. The benefit of this processing method is that it breaks down the sugars without any adverse sensory effects or change in functionality. Rao and Vakil (1983) found that as the dose of gamma-radiation increased, there was a greater decrease of raffinose content in *Phaseolus aureus*, from 15% at 1 kGy to 45% at 10 kGy. This trend was also observed with the stachyose and verbascose content in the *Phaseolus*

Table 2.4 Changes in Alpha-Galactoside Content in Pulses during Fermentation.

	Fermentation Parameters						Change in Alpha-Galactoside Content								
Pulse Name	Format	B:W[@] ratio (w/v)	Time (hr)	Temp.[#] (°C)	Ferm. Type	MO	Raffinose		Stachyose		Verbascose		Ciceritol		Reference
							Init.[$]	Final	Init.[$]	Final	Init.[$]	Final	Init.[$]	Final	
Lentils	flour	1:10	96	30	Nat.	Inherent	0.220 ±0.030	ND	1.930 ±0.180	ND	N/A		1.010 ±0.180	ND	(Vidal-Valverde et al. 1993)
Common Beans	flour	1:6	48	42	Nat.	Inherent	0.620 ±0.050	0.010 ±0.000	2.830 ±0.040	ND	0.190±0 .010	ND	N/A		(Granito et al. 2002)
		1:12	48	42	Nat.	Inherent	0.620 ±0.050	ND	2.830 ±0.040	ND	0.190±0 .010	ND	N/A		
	whole bean	1:12	48	42	Nat.	Inherent	0.620 ±0.050	ND	2.830 ±0.040	ND	0.190±0 .010	ND	N/A		
Blue Lupin	flour	1:2	20	30	Nat.	Inherent	1.970 ±0.290*	7.460 ±0.320*	15.610 ±0.900*	14.640 ±0.940*	7.010 ±0.460*	6.350 ±0.590*	N/A		(Kaczmarska et al. 2017)
					Ind.	*Lactobacillus delbrueckii subsp. bulgaricus Streptococcus thermophillus*	1.970 ±0.290*	4.940 ±0.320*	15.610 ±0.900*	14.640 ±0.940*	7.010 ±0.460*	4.980 ±0.570*	N/A		
Soybean	flour	1:2	20	30	Nat.	Inherent	3.190 ±0.0*0*	2.580 ±0.160*	14.100 ±0.550*	11.020 ±0.970*	0.660 ±0.000*	1.180 ±0.090*	N/A		
					Ind.	*Lactobacillus delbrueckii subsp. bulgaricus Streptococcus thermophillus*	3.190 ±0.000*	1.010 ±0.200*	14.100 ±0.550*	8.120 ±0.710*	0.660 ±0.000*	0.910 ±0.040*	N/A		
African yambean	flour	1:3	48	30	Nat.	Inherent	0.600 ±0.030*	0.120 ±0.010*	2.170 ±0.010*	0.200 ±0.020*	0.150 ±0.000*	0.120 ±0.020*	N/A		(Azeke et al. 2005)
					Ind.	*Lactobacillus plantarum*	0.600 ±0.030*	0.110 ±0.020*	2.170 ±0.010*	0.080 ±0.030*	0.150 ±0.000*	0.030 ±0.010*	N/A		
Cowpeas	flour	1:10	36	30	Ind.	*Rhizopus oligosporus*	0.520	ND	2.040	ND	N/A		N/A		(Ibrahim et al. 2002)
						Lactobacillus plantarum	0.520	ND	2.040	ND	N/A		N/A		

N/A indicates that this sugar was not tested for. @ = Bean:Water. w/v = weight to volume. # = Temperature. Nat. = Natural. Ind. = Induced. Inherent indicates that the pulse was fermented using the microbiota already present on the sample. Ferm. = Fermentation. MO = Microorganism. $ = Initial value. * = indicates value was converted from g/kg dry matter to % dry matter.

aureus. The products of this radiolytic breakdown were found to be glucose, galactose and melibiose (alpha-galactose (1-6) glucose), similar to the products obtained from enzymatic breakdown. Other studies have also reported a dose-dependent decrease in total alpha-galactosides when irradiating various pulses (Dixit et al. 2011; Tresina and Mohan 2011; Yun et al. 2012). Al-Kaisey et al. (2003) were able to achieve a 100% reduction in raffinose and stachyose in *Vicia faba* with a 7.5 kGy dose, and 100% reduction in verbascose with a 10 kGy dose. A possible reason for the paucity of material on this subject could be the public perception of irradiated food. Studies show that 46% of the Chilean population believe that irradiated food is equivalent to radioactive food, while 88% of the American population have heard of irradiation but do not really know much about it (Junqueira-Gonçalves et al. 2011; Resurreccion et al. 1995).

2.5.5 Combining methods

Several researchers have attempted to apply multiple processing methods in order to achieve a full (100%) reduction in alpha-galactoside content (Baik and Han 2012; Devindra et al. 2011; Machaiah and Pednekar 2002; Pugalenthi, Siddhuraju, and Vadivel 2006). The combination of processing methods ensures a more thorough removal of alpha-galactosides but comes at the cost of additional energy and resources expended. One of the more widely known processing combinations is soaking and cooking, common in households where the pulses are processed into a meal. By soaking the pulses before cooking, the efficacy of alpha-galactoside removal is improved (Baik and Han 2012). As mentioned before, an increase in alpha-galactoside content after cooking can be avoided when those pulses are presoaked. Machaiah and Pednekar (2002) combined germination of pulses and subsequent radiation and examined their combined effect on alpha-galactoside content and found that the greatest reduction occurred on the third day of

germination for all surveyed pulses (*Phaseolus aureus*, *Vicia faba*, *Cicer arietinum*, *Dolichos biflorus*, and *Vigna unguiculata*). While the addition of irradiation was able to reduce alpha-galactoside content further than just germination in the first and second day, by the fourth day all treatments had reduced alpha-galactoside content by >95% (Machaiah and Pednekar 2002). Devindra et al. (2011) also examined post germination treatments, by heat-treating the germinated *Cajanus cajan*, as a means to reduce the germination time. They saw that germination for 16 h reduced more alpha-galactosides compared to 8 h, and of the three methods (autoclaving, cooking and pressure cooking), cooking reduced the alpha-galactoside content the most (Devindra et al. 2011).

2.6 Conclusion

Although pulses are a good source of protein and dietary fiber, the presence of alpha-galactosides impair the inclusion of pulses in a regular diet. Various methods have been tested on their effectiveness to remove alpha-galactosides from different pulses. Soaking in water is a common household technique that is able to remove 20-70% of alpha-galactosides, given that the conditions are optimized. It is also an easy, low-cost method to use, however it uses up a large amount of resources. Cooking can remove 30-90% of alpha-galactosides, but sufficient removal only occurs if the pulses are presoaked first. Thermal degradation such as autoclaving, with 20-70% removal and extrusion, with 30-80% removal, confers mixed and sometimes increasing results, as alpha-galactosides are quite heat stable. Enzymatic degradation has proven to be more efficient at removing alpha-galactosides, with crude enzyme application, fermentation, and germination all achieving a near 100% removal of alpha-galactosides. Future studies should examine the

physiochemical and sensory properties of novel foods formulated with alpha-galactoside-removed pulse flours.

3 Materials and Methods

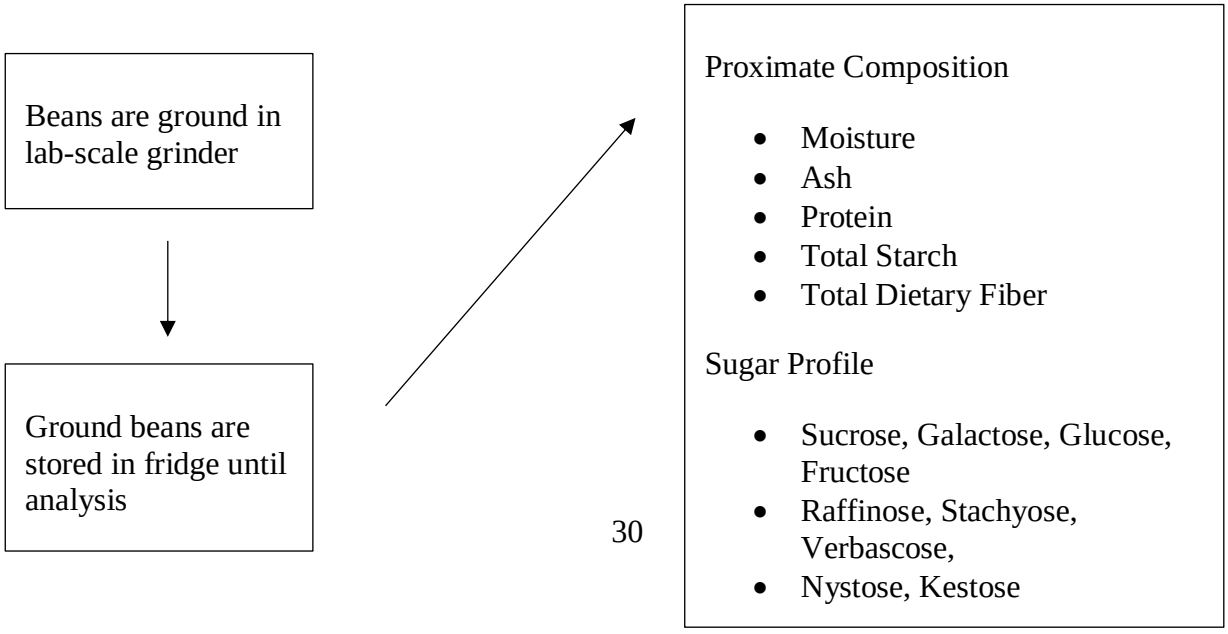
3.1 Chemical Composition of Ontario Beans

3.1.1 Materials

Six varieties of beans were obtained from the University of Guelph Bean Breeding Program in the Department of Plant Agriculture. These varieties were: Dark Red Kidney Bean (Dynasty), Light Red Kidney Bean (Inferno), Navy Bean (Rexeter), Cranberry Bean (Red Rider), Black Bean (Zenith), and White Kidney Bean (Yeti). Sugar standards and aspartic acid were purchased from Millipore Sigma (Burlington, MA, USA).

3.1.2 Bean Flour (BF) Preparation

Beans were crushed in a laboratory scale grinder (M 20 Universal Mill, IKA, Wilmington, USA), passed through a 500µm sieve and stored in sealed plastic bags at 4°C. Flour was sieved at 500µm since this was the standard found in previous literature.



3.1.3 Ash Content

Ash was determined according to AOAC method 923.03 by placing 2 grams of BF in a ceramic crucible and placing the crucible in a muffle furnace (Lindberg/Blue M, Thermo Scientific, USA). The crucibles were heated to 525ºC for 8 hours, and then taken out when the oven cooled down to 100ºC. The crucibles were placed in a desiccator and cooled until a constant weight was achieved. The crucible was weighed, and ash content was calculated using Equation (**1**)

$$Ash\ (\%) = \left(\frac{crucible_{ash} - crucible}{crucible_{bean} - crucible}\right) \times 100. \quad (1)$$

where $crucible_{ash}$ = the weight of the crucible with ash,

crucible = weight of the empty crucible, and

$crucible_{bean}$ = weight of the crucible with BF.

Ash content was reported as the average of triplicates.

3.1.4 Moisture Content

Moisture content was determined according to AOAC method 925.09 by placing 2 grams of BF in a dish and drying it in an oven (VWR, Pennsylvania, USA) for 5 hours at 105ºC. The dishes were then placed in a desiccator and cooled until a constant weight was achieved. The remaining dry BF was weighed, and moisture content was calculated using Equation 2.

$$Moisture\ (\%) = (\frac{crucible_{bean} - crucible_{dry\ bean}}{crucible_{bean} - crucible}) \times 100 \quad (2)$$

Where $crucible_{bean}$ = weight of crucible with BF,

$crucible_{dry\ bean}$ = weight of crucible with BF after drying in the oven, and

crucible = weight of empty crucible.

The moisture content was reported as the average of triplicates.

3.1.5 Protein Content

Protein was determined using the Dumas method, in line with AOAC method 997.02. Briefly, fifty milligrams of BF were placed in an aluminium crucible, which was then combusted at 900°C using a combustion oven (Flash 2000, Thermo Scientific, USA). The protein values were calculated using a conversion factor of 6.25 to convert nitrogen content to protein content. Aspartic acid was used as the standard.

3.1.6 Total Starch Content

Total starch content was determined using a Megazyme rapid analysis kit (Megazyme International, Ireland), according to AOAC method 996.11. In brief, 100 milligrams of BF were digested using alpha-amylase and amyloglucosidase (AMG) enzymes, which transformed the starch within the BF into glucose. The concentration of glucose was measured using a spectrophotometer (Evolution 60 UV-Vis, Thermo Scientific, USA) at a wavelength of 515nm, using the software VisionLite v2.2. Total starch was calculated using Equation 3.

$$\%S = \Delta E \times F \times \frac{100}{0.1} \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180} \quad (3)$$

where ΔE = absorbance,

F = conversion factor from absorbance to glucose,

100/0.1 = dilution factor,

1/1000 = conversion from µg to mg,

W = dry weight of sample, and

162/180 = conversion factor from free D-glucose to anhydro-D-glucose (as found in starch).

A solution of 100µg/mL glucose was used as standard. Measurements were performed in duplicate.

3.1.7 Total Dietary Fibre Content

Total dietary fibre content was measured gravimetrically using a Megazyme rapid analysis kit (Megazyme International, Ireland), according to AOAC method 991.42. Briefly, 1 gram of BF was digested with alpha-amylase, protease, and AMG to remove the protein and starch from the fibre. 95% ethanol preheated to 60ºC was used to precipitate the fibre (insoluble and soluble) from the sample, and the supernatant was removed by vacuum filtration (V-100, Büchi, Postfach, Switzerland). The fibre-containing crucible was dried overnight at 103ºC and weighed the next day. Protein and ash contents of each sample were also calculated according to previous methods. Total dietary fibre was calculated using Equation 4.

$$Total\ Dietary\ Fibre\ (\%) = \left(\frac{\frac{R_1 + R_2}{2} - P - A - B}{\frac{m_1 + m_2}{2}}\right) \times 100 \quad (4)$$

where R_1= fibre residue weight from sample m_1,

R_2= fibre residue weight from sample m_2,

A = ash weight from sample m_1,

P = protein weight from m_2, and

$$B = \frac{BR_1 + BR_2}{2} - BP - BA \quad (5)$$

where BR = blank residue,

BP = blank protein, and

BA = blank ash.

Samples were analyzed in duplicate.

3.1.8 High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) Sample Preparation

BF extracts were prepared according to the method explained by Bainy et al. (2008). One gram of BF was mixed with 10ml of 50% ethanol at room temperature for 30 min, using a stirring water bath (Basic RCT, IKA, Wilmington, USA). The mixture was centrifuged at 10000xg for 10 minutes, using a Beckman CS-15R centrifuge, and the supernatant was removed. The BF was extracted twice in this manner and the combined supernatant (20mL) was evaporated in a rotovap (Rotovapor R-205, Büchi, Postfach, Switzerland). The resulting extract (~5mL) was made to 10mL in a volumetric flask.

3.1.9 HPAEC-PAD Analysis of Sugars

HPAEC-PAD was used to determine the sugar profiles of the bean samples, using a modified method from Brummer, Kaviani and Tosh (2015). The sugars evaluated were sucrose, glucose, galactose, fructose, raffinose, stachyose, verbascose, nystose and kestose. Raffinose, stachyose and verbascose are the prevalent alpha-galactoside sugars found in beans, while nystose and kestose are fructo-oligosaccharides that are considered FODMAPS. Prior research has shown that germination influences the levels of sucrose, glucose, galactose and fructose, thus these sugars were included in the sugar profile analysis. The samples were analyzed using HPAEC-PAD (DX600, Thermo Scientific Dionex, Sunnyvale, USA), using a CarboPac PA1 column (4 × 250 mm) and guard (3 × 25 mm). Samples were eluted in 7mM NaOH for 25min, then with a gradient to 100mM NaOH over 25min, then 10min at 300mM NaOH. Sugar standards containing all 9 sugars were made at concentrations of 1, 15, 30, 50, 80, and 100 µg/mL to quantify the sample sugar profiles. Pulse potentials (E, volts) and durations (t, ms) were $E_1 = 0.05$ and $t_1 = 480$; $E_2 = 0.6$ and $t_2 = 180$; $E_3 = -0.6$ and $t_3 = 60$; with a 1.0 s detector response time. Samples were run in triplicate at two dilutions (0.1 and 0.01) to capture all sugar peaks.

3.1.10 Statistical Analysis

The statistical analysis for bean composition was performed by one-way ANOVA and Fisher's LSD test (to determine the difference between means), using R Commander. A 95% confidence interval was used for statistical significance.

3.2 Optimization of Bean Germination

3.2.1 Experimental Design

Response surface methodology is a statistical method that takes data from an experimental design and uses it to develop a multivariate equation, that can describe the effect of independent process variables on a certain outcome. In this study, the process of germination was influenced by three variables: duration of germination (X_1), ambient temperature during germination (X_2), and light exposure (X_3). The relationship between these variables and the response (Y) is described in Equation 6.

$$Y = f(X_1, X_2, X_3) \tag{6}$$

A central composite design (CCD) was chosen since it could efficiently determine the effect of process variables (time, temperature, light) on the desired responses (total alpha-galactosides, etc.) in a minimum number of experimental runs (Krishnaswamy et al. 2013).

The levels chosen for each variable were meant to simulate the full range of conditions that would be ideal for germinated beans. Table 3.1 displays the levels selected for each process variable. Note that for light duration, 12 hours of light meant that the chamber would be lit for the first 12 hours of a 24-hour cycle, followed by 12 hours of darkness, and the cycle would repeat every 24 hours.

Table 3.1 Independent variables and both their coded and uncoded levels used for the CCD.

Independent Variables	**Coded**	**Units**	**Coded Levels**		
			-1	**0**	**1**
Germination Duration	X_1	hrs	24	48	72

Ambient Temperature	X_2	^oC	22.5	27.5	32.5
Light Duration per 24 hrs	X_3	hrs	0	12	24

Using Design Expert software v11.1.1.0 (Stat-Ease Inc., Minneapolis, MN, USA), 20 experimental runs were identified. This included 8 factorial points, 6 axial points, and 6 repeated centre points, all of which are displayed in Table 3.2.

Table 3.2 Central composite design showing the experimental combinations of the different coded levels of light exposure, ambient temperature and germination duration.

Experiment no.	Duration (hrs)	Temperature (^oC)	Light Exposure (hrs)	Type of point
1	24	22.5	0	Factorial points
2	72	22.5	0	
3	24	32.5	0	
4	72	32.5	0	
5	24	22.5	24	
6	72	22.5	24	
7	24	32.5	24	
8	72	32.5	24	
9	24	27.5	12	Axial points
10	72	27.5	12	
11	48	22.5	12	
12	48	32.5	12	
13	48	27.5	0	
14	48	27.5	24	
15-20	48	27.5	12	Centre points

The data from these experimental runs were used to develop a regression equation in the form of a second-order polynomial, as shown in Equation 7.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i>j}^{k} \beta_{ij} X_i X_j \quad (7)$$

Where Y is the predicted response,

β_0 is the model y-intercept,

β_i, β_{ii}, and β_{ij} are the regression coefficients of the linear, quadratic and bilinear effects respectively,

X_i and X_j are the coded independent variables, and

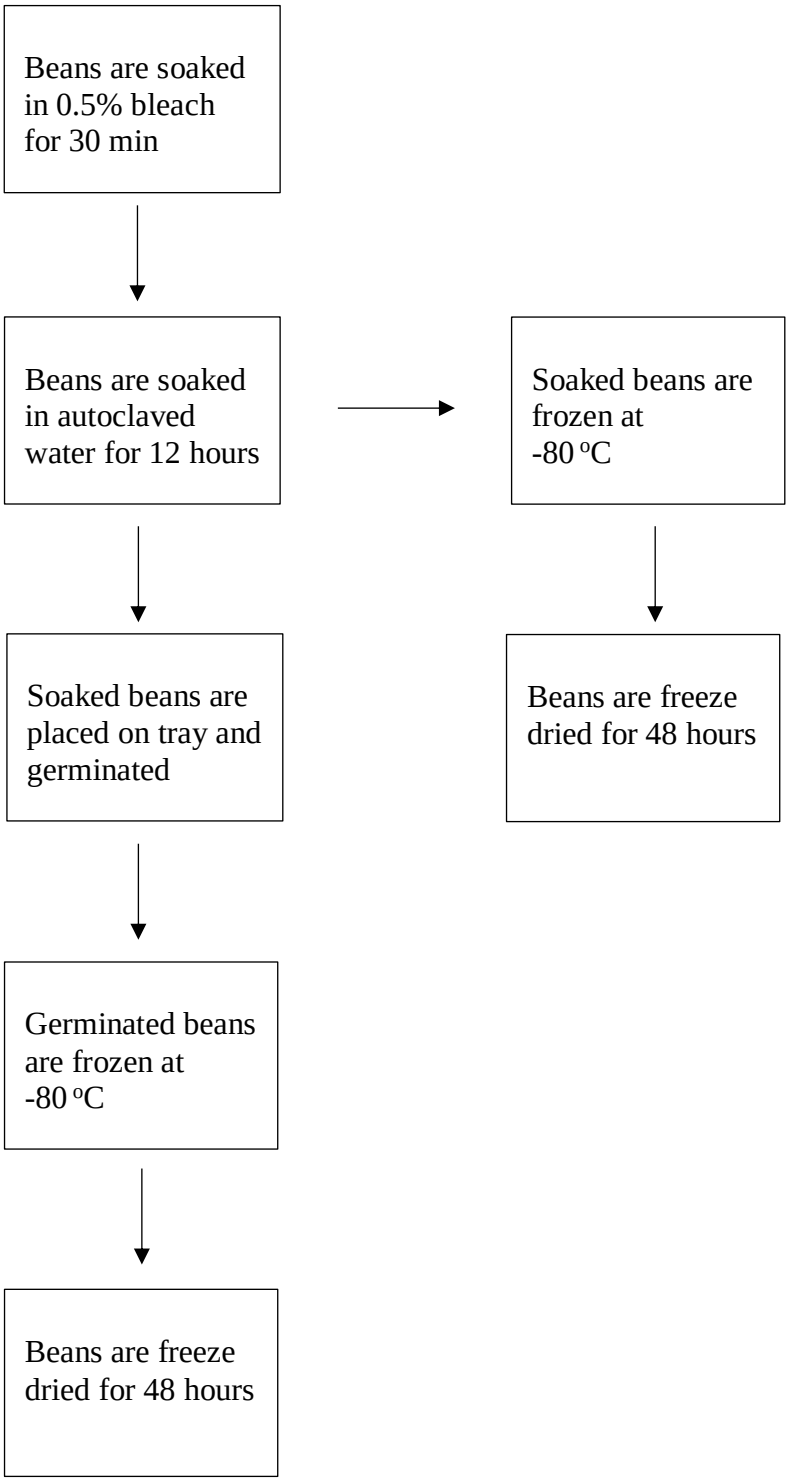
k is the number of independent variables (in this case, 3).

3.2.2 Materials

Germination paper was purchased from VWR (Radnor, PA, USA), and bleach was purchased from Millipore Sigma (Burlington, MA, USA).

3.2.3 Germination of Dynasty Beans

Germination of beans was conducted using a modified method from Sangronis and Machado (2007). 150g of beans were soaked in 250 mL of 0.5% bleach for 30 min to sterilize the seed coats, then rinsed with autoclaved water until the pH of the rinsed water reached 7. 600mL of autoclaved water was added to the beans and the beans were left to soak for 12 hours. Soaked beans were placed in a germination tray that was lined with damp germination paper. The trays were placed in germination chambers and left to sprout under the specified conditions of the experimental run. Every day, beans were moistened with water and moldy beans were removed. After germination, the sprouted beans were collected and frozen at –80^{o}C for at least 1 day. The germinated beans were then freeze dried at -40^{o}C for 48 hours using a freeze drier (VirTis Genesis 25, SP Industries, Warminster, PA, USA). The resulting dried germinated beans were ground into flour, passed through a 500µm sieve, and kept in a desiccator at room temperature. Flour was sieved at 500µm since this was the standard found in previous literature. Beans that were only soaked and not sprouted were also freeze dried and ground into powder to compare the difference between raw and soaked beans.


Beans are soaked in 0.5% bleach for 30 min
Beans are soaked in autoclaved water for 12 hours
Soaked beans are frozen at -80 °C
Soaked beans are placed on tray and germinated
Beans are freeze dried for 48 hours
Germinated beans are frozen at -80 °C
Beans are freeze dried for 48 hours

3.3 Proximate and Nutritional Analysis of Germinated Bean Flour (GBF)

3.3.1 Materials

GBF was produced as mentioned above. Trypsin, chymotrypsin, protease, Folin Ciocalteu's (FC) reagent, DPPH radical, ABTS radical, sodium persulfate, were obtained from Millipore Sigma (Burlington, MA, USA).

3.3.2 Moisture

Moisture was conducted as previously described in section 3.1.4.

3.3.3 Protein

Protein was conducted as previously described in section 3.1.5.

3.3.4 Protein Digestibility

In-vitro protein digestibility was calculated using the method modified from Nosworthy et al. (2017). Briefly, an equivalent amount of GBF that contained 62.5 mg of protein was hydrated in 10mL of 37°C milli-Q water for 1 hour. Then the pH of the sample was adjusted to 8.0 with 0.1 M NaOH and/or HCl and the sample was left to equilibrate for 30 min. Afterwards, the pH was again adjusted to 8.0 using 0.1 M NaOH and/or HCl. 25 millilitres of a multi-enzyme solution was prepared consisting of 40 mg of trypsin, 77.5 mg of chymotrypsin and 32.5 mg protease. The multi-enzyme solution was prepared fresh on the day of analysis and kept at 37°C for 20 minutes, before adjusting the pH to around 8.0 with 0.1 M NaOH and/or HCl. The multienzyme solution

was immediately placed in an ice bath after adjustment and kept there until use. The solution was made fresh each day immediately prior to analysis. Upon rehydration, 1 mL of the multi-enzyme solution was added to the 10-mL sample dispersion, and the pH of the solution was recorded every 60 s for 10 min using a pH meter (Orion 525A+, Thermo Scientific, USA). The change in pH at 10 min of digestion was used to calculate the % *in-vitro* protein digestibility (IVPD) of the samples, using Equation 8.

$$IVPD\ (\%) = 65.66 + 18.10 \times \Delta_{ph} \quad (8)$$

where Δ_{ph} is pH (at 10 minutes) – pH (at 0 minutes).

3.3.5 Resistant Starch

Resistant starch (RS) was quantified using the Resistant Starch Kit (Megazyme International, Ireland) and a modified method. Briefly, 100mg of GBF was placed in a tube with 4mL of pancreatic alpha-amylase (10 mg/mL) containing AMG (3 U/mL) and blended using a vortex mixer. The tube was placed in a shaking water bath (SW22, Julabo, Germany) at 37°C and incubated for 16 hours, while shaking at 200 strokes/min. After incubation, 4mL of 99% ethanol was added to stop the enzymatic reaction, and tubes were centrifuged at 1500xg for 10 min (CL2, Thermo Scientific, USA). The supernatant was saved, and pellets were resuspended in 2mL of 50% ethanol. Another 3mL of 50% ethanol was added and tubes were centrifuged again under same conditions. This process was performed twice, and both supernatants were saved. The supernatant was made up to 25mL in a volumetric flask using 50% ethanol. The remaining pellet of RS was digested with 2M KOH for 20 min at 0°C, after which 8 mL of 1.2 M sodium acetate buffer (pH 3.8) and 0.1 mL of AMG (3,300 U/mL) was added and tubes placed in a 50°C water bath for 30 min. Digested RS solutions were diluted and both RS and supernatant (containing

digestible starch (DS)) were incubated with GOPOD reagent for 30 min. The resulting pink solutions were read in a spectrophotometer (Evolution 60 UV-Vis, Thermo Scientific, USA) at a wavelength of 515nm, using the software VisionLite v2.2, to quantify glucose. RS and DS were calculated using Equation (**3**) A solution of 100µg/mL glucose was used as standard. Measurements were performed in quadruplicate.

3.3.6 HPAEC-PAD Sample Preparation and Analysis

Sample preparation and analysis was performed as previously described in sections 3.1.8. and 3.1.9.

3.3.7 TPC Sample Preparation

To prepare sample extracts for TPC analysis, a method modified from Aguilera et al. (2010) was used. 0.5g of GBF was mixed with 10mL of 70% methanol containing 1% HCl at 50°C for 30min. The addition of acid facilitates the removal of bound phenolic acids. The mixture was centrifuged at 10,000xg for 10 min and the supernatant was removed. The procedure was performed twice on the same GBF sample and the combined supernatant (20mL) was made up to 25mL in a volumetric flask.

3.3.8 TPC Analysis

TPC was quantified using the method from Padhi et al. (2017). FC reagent was diluted ten-fold to produce 0.2N of FC reagent. 25µL aliquots of sample/standard was pipetted in triplicate in a 96-well microplate. 125µL of 0.2N FC reagent was added to each well and left to react in the dark. After 10 min, 125µL of 7.5% sodium carbonate solution was added to each well and the plate was swirled slightly to mix the solutions. The plate was left to react in the dark for 30 min. The

absorbance was read at 765nm using a microplate reader (Powerwave XS2, Biotek, USA), and analyzed with the software Gen5 v1.11. Gallic acid at concentrations of 31.25, 62.5, 125, 250 and 500 µg/mL were used as standards.

3.3.9 Antioxidant Capacity Sample Preparation

To prepare sample extracts for both ABTS and DPPH antioxidant capacity analyses, a method modified from Aguilera et al. (2010) was used. 0.5g of GBF was mixed with 4mL 70% methanol at 50°C for 30min. Acid was not used as the DPPH assay is sensitive to acid (Shalaby and Shanab 2013). The mixture was centrifuged at 10,000xg for 10 min and the supernatant was removed. The procedure was performed twice on the same GBF powder and the combined supernatant (8mL) was made up to 10mL in a volumetric flask.

3.3.10 DPPH Antioxidant Capacity Analysis

The method used for DPPH analysis was modified from Padhi et al. (2017). DPPH stock solution was made by dissolving 34.5mg of DPPH powder in 10mL of 100% methanol and stirred for at least 15 minutes. The DPPH working solution was prepared by diluting 10mL of stock solution to 100mL using a volumetric flask. Trolox solution was prepared by dissolving 25mg of Trolox powder in 5mL of 100% methanol to create a 20mM stock solution. This stock solution was made fresh daily and diluted to make the standards at 62.5, 125, 250, 500, 750, and 1000 µM. 25µL aliquots of sample/standard was pipetted in triplicate in a 96-well microplate. 200µL of DPPH radical solution was added to each well and the plate was left to react in the dark for 60 min. The absorbance was read at 517nm using the microplate reader (Powerwave XS2, Biotek, USA), and analyzed with the software Gen5 v1.11. 70% methanol was used as blank and control.

3.3.11 ABTS Antioxidant Capacity Analysis

The method used for ABTS analysis was modified from Thaipong et al. (2006). ABTS radical stock solution was made by mixing equal amounts 7.4mM sodium persulfate and 2.6mM ABTS, and letting the mixture react overnight for 16 hours. ABTS radical working solution was made by diluting 5mL of stock solution with 85mL of milli-Q water. Trolox solution was made as described in section 3.3.10, and diluted to make the standards at 62.5, 125, 250, 500, 750, and 1000 µM. 10µL of sample/standard was pipetted in triplicate in a 96-well microplate. 190uL of ABTS radical solution was added to each well and left to react in the dark for 120 min. The absorbance was read at 734nm using the microplate reader (Powerwave XS2, Biotek, USA), and analyzed with the software Gen5 v1.11. Milli-Q water was used as blank and control.

3.3.12 Statistical Analysis

Design Expert software v11.1.1.0 (Stat-Ease Inc., Minneapolis, MN, USA) was used to perform the ANOVA for all models and generate the response surface plots. The difference between means was conducted using R Commander for Fisher's LSD. A 95% confidence interval was used for statistical significance.

4 Results and Discussion

4.1 Chemical Composition of Ontario Beans

4.1.1 Proximate Analysis

The results of the proximate analysis are outlined in Table 4.1. While moisture, protein, ash and total starch varied significantly between classes of beans, total dietary fibre was not significantly

different among the six classes. Fat was not analyzed because beans typically contain less than 2% fat, and thus it was not relevant for this thesis to measure the fat content.

Table 4.1 Proximate analysis of six Ontario beans.

Bean Market Class	Moisture (%db)	Protein (%db)	Ash (%db)	Total Starch (%db)	TDF* (%db)
Inferno	12.88**±0.00[a]	23.67±0.40[c]	4.30±0.07[b]	38.21±0.43[a]	20.25±0.02[a]
Zenith	9.89±0.00[b]	23.66±0.07[c]	4.45±0.03[b]	33.65±0.52[cd]	22.02±0.71[a]
Dynasty	11.96±0.00[ab]	24.68±0.11[b]	4.89±0.12[ab]	35.44±0.90[bc]	21.40±0.34[a]
Rexeter	10.39±0.00[ab]	19.71±0.11[d]	5.15±0.05[a]	36.98±0.44[ab]	20.85±2.48[a]
Red Rider	10.92±0.00[ab]	24.08±0.08[bc]	4.58±0.02[ab]	33.66±0.56[cd]	20.12±1.32[a]
Yeti	9.52±0.00[b]	26.85±0.54[a]	4.89±0.09[ab]	31.75±0.94[d]	21.66±0.87[a]

*Total Dietary Fibre

**Means are recorded as average ± standard deviation. N=3 except for Total Starch and Total Dietary Fibre where N=2. Means with the same letter in a column are not significantly different.

The proximate composition values of the six classes ranged from 9.52% (Yeti) to 12.88% (Inferno), from 19.71% (Rexeter) to 26.85% (Yeti), from 4.30% (Inferno) to 5.15% (Rexeter), from 31.75% (Yeti) to 38.21% (Inferno), from 20.12% (Red Rider) to 22.02% (Zenith), respectively for moisture, protein, ash, total starch, and TDF.

Moisture content values observed were in line with moisture content of dark red kidney, light red kidney and navy beans obtained from Chung et al. (2008) (9.1-10.3%) and roba, awash and beshbesh beans from Shimelis and Rakshit (2007) (9.08-11.00%). Protein values were lower than black, cranberry, great northern, light red kidney, navy and pinto beans reported by Wang et al. (2017) (24.4-27.7%), higher than roba, awash and beshbesh beans reported by Shimelis and Rakshit (2007) (17.96-22.07%), and in line with dark red kidney, light red kidney and navy beans reported by Chung et al. (2008) (23.1-26.6%). Ash content values for these market classes of beans were higher than dimeta, napirira and nanyati beans reported by Fan and Beta (2016) (3.57-3.77%), curruquila and almonga beans from Pedrosa et al. (2015) (3.65-4.64%), and roba, awash and beshbesh beans from Shimelis and Rakshit (2007) (2.86-4.26%), but were in line with black,

cranberry, great northern, light red kidney, navy and pinto beans reported by Wang et al. (2017) (3.84-5.19%). In the 6 market classes profiled here, only Rexeter was siginificantly different from Inferno and Zenith beans in terms of ash content.

The total starch values were lower than reported for navy, pinto, black, cranberry and light red kidney beans (36.0-40.2%, Wang et al. 2017), but in line with reported values for black beans (33.6-36.3%, Vargas-Torres et al. 2004). TDF content was found to be lower than previously reported values by Kutoš et al. (2003), Granito et al. (2002), Dueñas et al. (2016) and Ramírez-Jiménez et al. (2014) (23.3-38.2%), but in line with values reported by Pedrosa et al. (2015) (18.50-20.05%). Among the market classes, TDF was not significantly different. One point to mention is that the TDF method used was a simple method, and thus constituents such as resistant starch, alpha-galactosides, and pectins were not fully captured in the TDF value, leading to lower values than what was previously published.

4.1.2 Sugar Profile

There are a variety of sugars found in common beans, such as glucose and sucrose, that contribute to the taste of these beans. Previous research has also confirmed the presence of alpha-galactosides such as raffinose, stachyose and verbascose in common beans (Silva and Braga 1982; Wang et al. 2010). Galactose and fructose were quantified in order to compare with the GBF later on in the thesis, while nystose and kestose were quantified to see if the fructo-oligosaccharide content would also be affected by germination.

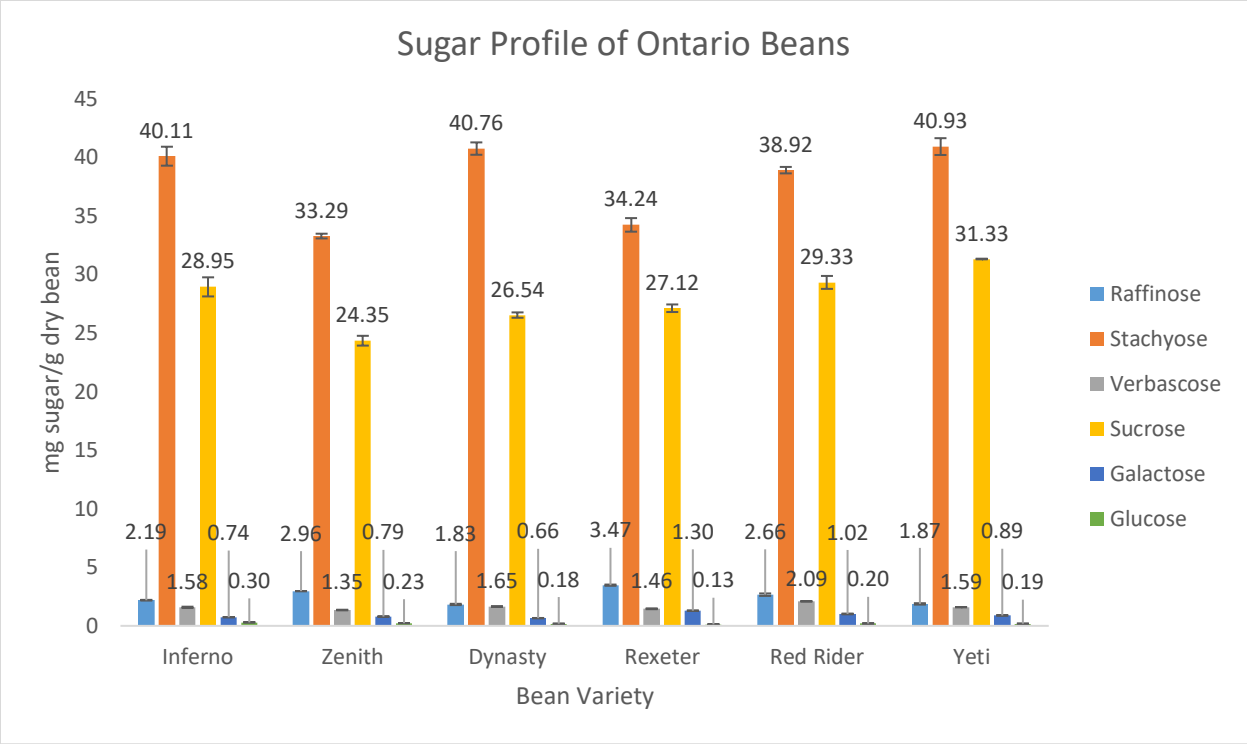


Figure 4.1 Sugar composition of 6 market classes of common beans (n=3)

Fructose, nystose and kestose were not detected in all 6 market classes.

The sugar composition of the 6 market classes are as shown in

. Values for alpha-galactosides ranged from 1.83 mg/g (Dynasty) to 3.47 mg/g (Rexeter) for raffinose, from 33.29 mg/g (Zenith) to 40.93 mg/g (Yeti) for stachyose, and from 1.35 mg/g (Zenith) to 2.09 mg/g (Red Rider) for verbascose. Values for soluble sugars ranged from 24.35 mg/g (Zenith) to 31.33 mg/g (Yeti) for sucrose, from 0.66 mg/g (Dynasty) to 1.30 mg/g (Rexeter) for galactose, and from 0.13 mg/g (Rexeter) to 0.30 mg/g (Inferno) for glucose. The order of sugars from greatest to least was the same for all market classes: stachyose > sucrose > raffinose > verbascose > galactose > glucose. This is in direct contrast to the proposed hypothesis, which stated that each bean would have a significantly different sugar profile. The hypothesis was built off of each variety being bred differently for differing purposes, thus there would have been a different sugar profile for each bean. However, all beans showed the same pattern in sugar content.

Fructose was not detected in any of the market classes, which aligns with a previous study by Sánchez-Mata et al. (1998). However, these results are contrary to results published by Granito et al. (2002) (1.2 mg/g fructose in Victoria class beans) and Phillips and Abbey (1989) (2.20-5.32 mg/g in kidney, black turtle, pinto, navy and great northern beans). These differences can be attributed to variances in methodology, variances between beans and reagents used in the experiment. Another reason for these differences is that the extraction method used in this thesis was not optimized and may not have extracted all the sugars fully. While a 50/50 ethanol/water solution was used to extract sugars, it may have been better to use different concentrations of ethanol, and a more aggressive extraction, to fully extract all the different sugars. Nystose and kestose were also not detected in any of the market classes.

Sucrose values were in alignment with previously published values of 37.75 mg/g for great northern beans (Sathe et al. 1983) and 36.6 mg/g for Victoria variety beans (Granito et al. 2002), but were higher than sucrose values found for black beans (22.7 mg/g, Granito and Álvarez 2006) and for kidney, black turtle, pinto, navy and great northern beans (7.90 to 17.71 mg/g, Phillips and Abbey 1989). Within the analyzed varieties, only Yeti and Zenith had significantly different sucrose contents.

Galactose was detected and quantified, unlike prior studies which did not detect any galactose in common beans (Granito et al. 2002; Granito and Álvarez 2006). The galactose content of Rexeter was significantly different from Inferno and Dynasty. Glucose was lower than values reported by Granito and Álvarez (2006) (1.7 mg/g) and Granito et al. (2002) (0.4 mg/g).

Table 4.2 Individual and Total Alpha-Galactosides in Beans

Bean Market Class	Raffinose (mg/g dry bean)	Stachyose (mg/g dry bean)	Verbascose (mg/g dry bean)	Total Alpha Galactosides (mg/g dry bean)
Inferno	2.19*±0.03^{d}	40.11±0.81^{ab}	1.58±0.06^{b}	43.88±0.84^{a}
Zenith	2.96±0.00^{b}	33.29±0.20^{c}	1.35±0.03^{d}	37.60±0.22^{b}
Dynasty	1.83±0.05^{e}	40.76±0.53^{a}	1.65±0.02^{b}	44.24±0.50^{a}
Rexeter	3.47±0.05^{a}	34.24±0.58^{c}	1.46±0.04^{c}	39.17±0.57^{b}
Red Rider	2.66±0.11^{c}	38.92±0.28^{b}	2.09±0.02^{a}	43.67±0.40^{a}
Yeti	1.87±0.06^{e}	40.93±0.72^{a}	1.59±0.01^{b}	44.40±0.72^{a}

*Means are recorded as average ± standard deviation, and N=3. Means with the same letter in a column are not significantly different.

The total alpha-galactoside content differed significantly between two groups of market classes (Table 4.2), with Inferno, Dynasty, Red Rider and Yeti having a significantly high total alpha-galactoside content than Zenith and Rexeter. Stachyose was the predominant alpha-galactoside, as confirmed by prior research (Obendorf and Górecki 2012). Raffinose content was lower than results reported by Phillips and Abbey (1989) for kidney, black turtle, pinto, navy and great northern beans (2.48-4.19 mg/g), Iyer et al. (1980) for great northern, kidney and pinto beans (5.6-9.3 mg/g), and Kelkar et al. (2012) for navy and pinto beans (7.75-9.25 mg/g). Stachyose content was found to be higher than results reported by Iyer et al. (1980) for great northern, kidney and pinto beans (24.0-29.5 mg/g), Barampama and Sinard (1993) for A321, A410, Calima and Dore de Kirundo beans (15.58-22.86 mg/g), Kelkar et al. (2012) for navy and pinto beans (26.00-30.00 mg/g), and Phillips and Abbey (1989) for kidney, black turtle, pinto, navy and great northern beans (10.17-16.03 mg/g). Verbascose content was found to be higher than previous results by Phillips and Abbey (1989), Iyer et al. (1980), Sathe and Salunkhe (1981) and Vidal-Valverde et al. (2002b), who all reported non-detectable levels of verbascose in different market classes of common beans.

The difference between the levels of alpha-galactosides in this study, compared to other studies, can be attributed to several factors. Since the beans used in this study were different from beans

analyzed in previous research, environmental factors such as soil composition, climate, moisture levels, and nutrients obtained during growth all affect the sugar profile of the beans. The maturation of the beans is another factor in differing sugar profiles. Prior research has demonstrated that as legumes mature in the pod, alpha-galactosides accumulate in the seed (Juana Frias et al. 1996; Górecki et al. 1996; Horbowicz and Obendorf 1994; Lowell and Kuo 1989). While stachyose and verbascose levels continue to increase during maturation, the rate of increase for raffinose is much smaller, due to raffinose being used as a substrate for higher order alpha-galactosides. This trend has been observed for peas, soybeans, and faba beans (Frías et al. 1996; Lowell and Kuo 1989; Saravitz, Pharr, and Carter 1987). In addition, alpha-galactosides accumulate very late in the maturation cycle, with verbascose accumulating after raffinose and stachyose (Obendorf and Górecki 2012). The beans used in this study could have been harvested at a later stage of maturation than the legumes used in the cited studies, leading to a difference in total alpha-galactosides.

Another factor in the difference between alpha-galactoside contents is the maturation conditions of the beans. One of the functions of alpha-galactosides is to enable the seed to tolerate desiccation. Blackman, Obendorf, and Leopold (1992) found that when soybeans matured under 100% humidity, there was an absence of stachyose and raffinose, a stark contrast to the high accumulation of stachyose and raffinose in desiccated soybean seeds.

4.1.3 Selection of Market Class for Germination

The preliminary research was conducted to determine which market class would undergo germination. The selection was made primarily on total alpha-galactoside content, followed by feasibility of nutritional improvement and potential for consumption in the Canadian market.

When comparing the total alpha-galactoside content, market classes Dynasty, Inferno, Red Rider and Yeti were not significantly different. Among these four classes, Dynasty was chosen because of its popularity in Canadian diets, as red kidney beans are preferred compared to other market classes of beans (Mudryj et al. 2012; Singh 2013).

4.2 Optimization of Bean Germination

4.2.1 Model fitting

To evaluate the validity of a model, several statistics were evaluated. The model should have a p-value under 5%, while the lack of fit statistic should have a p-value over 5%. The coefficient of determination (R^2) measures the proportion of variation in the response that is not attributed to random error and is a reliable indicator of model fit, thus it was used to evalutate the goodness of the models. An acceptable R^2 value for a good model fit is ≥ 0.8, but an R^2 value of ≥ 0.6 can be accepted when conducting preliminary analyses (Joglekar and May 1987; Malcolmson, Matsuo, and Balshaw 1993). Since R^2 values increase when more terms are added to a model (Dahmoune et al. 2015), and thus the model could have a poor prediction of the response (Myers, Montgomery, and Anderson-Cook 2009), a comparison between R^2 and adjusted R^2 was used to provide a better estimate of the model's fit, with the difference being not more than 0.2 (DiNardo, Subramanian, and Singh 2018).

4.2.2 Sugar Profile

When comparing raw and soaked Dynasty beans in Figure 4.2, there was a significant decrease in stachyose and sucrose, but a significant increase in raffinose, verbascose and fructose.

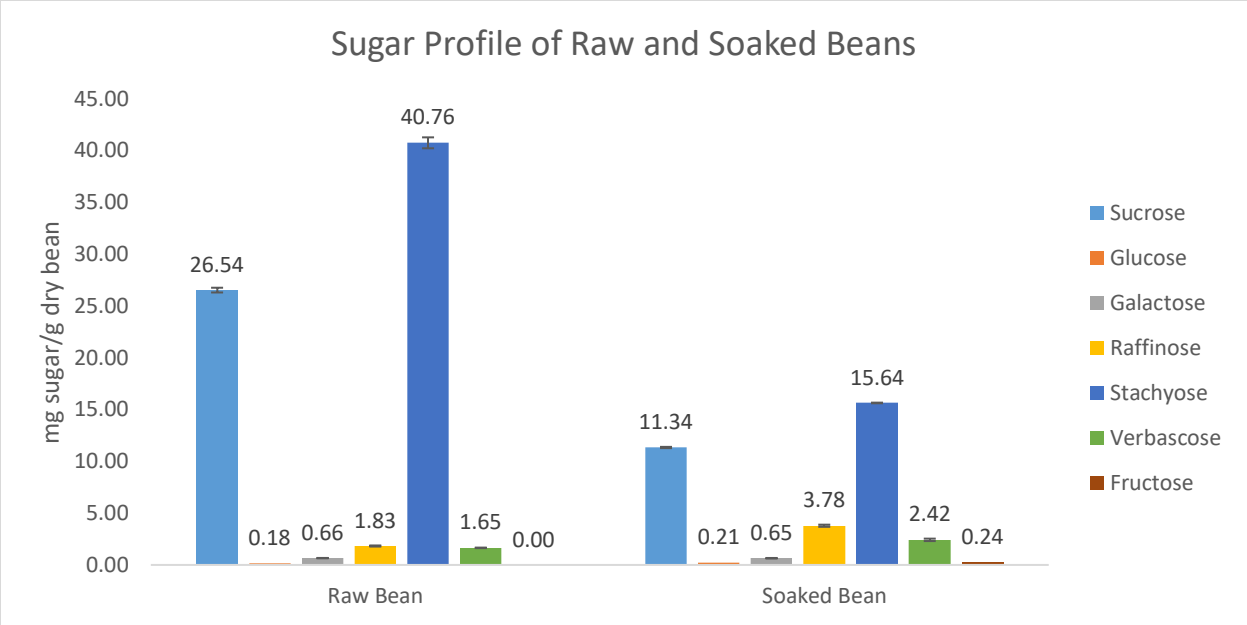


Figure 4.2 Sugar profile in raw and soaked Dynasty beans

The increase in raffinose and verbascose after soaking is contrary to published research, suggesting that there could be a large variance in sugar content within the full bag of beans (Aguilera et al. 2009; Devindra and Aruna 2017; Egounlety and Aworh 2003; Oboh et al. 2000). Fructose content was generated during soaking, suggesting the moisture-mediated activation of enzymes in the bean that cleave sugars into their constituent units. Nystose and kestose were not detected in any of the raw, soaked, or germinated samples.

4.2.2.1 Alpha-galactosides

Table 4.3 summarizes the raffinose, stachyose, verbascose, and total alpha-galactoside contents in the 20 trials.

Table 4.3 Experimental data for raffinose, stachyose, verbascose, and total alpha-galactoside content for the three variable, 3 level CCD analysis.

Trial	Factors*			Responses			
	X_1	X_2	X_3	Raffinose (mg/g dry bean)	Stachyose (mg/g dry bean)	Verbascose (mg/g dry bean)	Total Alpha-Galactosides (mg/g dry bean)
1	-1	-1	-1	3.92**±0.10^{abcd}	28.82±0.68^{ab}	2.00±0.04^{abc}	34.73±0.65^{ab}
2	1	-1	-1	2.03±0.06^{bcd}	4.53±0.04^{c}	0.84±0.03^{abcd}	7.39±0.10^{bc}
3	-1	1	-1	8.35±0.08^{a}	18.25±0.56^{abc}	2.16±0.04^{ab}	28.75±0.66^{abc}
4	1	1	-1	2.22±0.04^{bcd}	1.59±0.04^{c}	0.30±0.00^{d}	4.11±0.01^{c}
5	-1	-1	1	4.55±0.04^{abcd}	35.85±1.06^{a}	2.38±0.02^{a}	42.78±1.03^{a}
6	1	-1	1	2.77±0.05^{bcd}	6.27±0.23^{bc}	0.85±0.01^{abcd}	9.90±0.19^{bc}
7	-1	1	1	6.05±0.11^{abc}	6.70±0.07^{bc}	1.84±0.03^{abcd}	14.59±0.08^{abc}
8	1	1	1	1.70±0.04^{bcd}	3.38±0.05^{c}	0.77±0.01^{bcd}	5.85±0.07^{c}
9	-1	0	0	6.17±0.11^{ab}	17.41±0.21^{abc}	1.84±0.05^{abcd}	25.42±0.14^{abc}
10	1	0	0	1.69±0.02^{bcd}	1.07±0.04^{c}	0.84±0.04^{abcd}	3.60±0.02^{c}
11	0	-1	0	2.14±0.15^{bcd}	4.43±0.11^{c}	0.69±0.04^{bcd}	7.26±0.30^{bc}
12	0	1	0	3.71±0.06^{abcd}	7.06±0.16^{bc}	1.04±0.02^{abcd}	11.81±0.14^{bc}
13	0	0	-1	2.67±0.04^{bcd}	6.02±0.08^{bc}	0.95±0.04^{abcd}	9.64±0.05^{bc}
14	0	0	1	1.20±0.03^{bcd}	1.68±0.01^{c}	0.39±0.02^{d}	3.27±0.05^{c}
15	0	0	0	3.46±0.03^{abcd}	9.18±0.05^{bc}	0.86±0.01^{abcd}	13.51±0.07^{bc}
16	0	0	0	0.87±0.02^{d}	1.55±0.02^{c}	0.45±0.02^{cd}	2.87±0.05^{c}
17	0	0	0	1.78±0.06^{bcd}	1.49±0.03^{c}	0.45±0.01^{d}	3.72±0.10^{c}
18	0	0	0	2.40±0.04^{bcd}	2.89±0.07^{c}	0.55±0.01^{cd}	5.85±0.12^{c}
19	0	0	0	1.34±0.04^{bcd}	0.38±0.02^{c}	0.51±0.02^{cd}	2.23±0.04^{c}
20	0	0	0	1.04±0.04^{cd}	1.39±0.01^{c}	0.44±0.03^{d}	2.88±0.05^{c}

*X_1=Time, X_2=Temperature, X_3=Light.
**Means are recorded as average ± standard deviation, and N=3. Means with the same letter in a column are not significantly different.

RSM was used to analyze the effect of the linear, interaction, and quadratic effects on the three factors, however only the significant parameters were used to generate the predictive model for each sugar. The ANOVA analyses of each predictive model are displayed in

Table 4.4.

Table 4.4 ANOVA for the CCD analyses for alpha-galactosides, with significant terms included.

Source	df*	Sum of Squares	Mean Square	F-value	p-value	Coefficient Estimate
Raffinose (mg/g dry bean) (R^2=0.8462, R^2 Adj**=0.8052, CV#=29.00%)						
Intercept						2.06
A-Time	1	34.67	34.67	45.71	< 0.0001	-1.86
B-Temp	1	4.37	4.37	5.76	0.0298	0.6609
AB	1	5.80	5.80	7.64	0.0144	-0.8513
A^2	1	17.76	17.76	23.42	0.0002	1.88
Model	4	62.60	15.65	20.63	< 0.0001	
Residual	15	11.38	0.7584			
Lack of Fit	10	6.61	0.6608	0.6929	0.7099	
Pure Error	5	4.77	0.9537			
Total	19	73.97				
Stachyose (mg/g dry bean) (R^2=0.8479, R^2 Adj=0.8073, CV=53.45%)						
Intercept						3.61
A-Time	1	813.71	813.71	44.55	< 0.0001	-9.02
B-Temp	1	184.28	184.28	10.09	0.0063	-4.29
AB	1	143.56	143.56	7.86	0.0134	4.24
A^2	1	385.40	385.40	21.10	0.0004	8.78
Model	4	1526.95	381.74	20.90	< 0.0001	
Residual	15	274.01	18.27			
Lack of Fit	10	222.12	22.21	2.14	0.2074	
Pure Error	5	51.89	10.38			
Total	19	1800.96				
Verbascose (mg/g dry bean) (R^2=0.8836, R^2 Adj=0.8699, CV=23.36%)						
Intercept						0.6337
A-Time	1	4.35	4.35	78.55	< 0.0001	-0.6598
A^2	1	2.80	2.80	50.49	< 0.0001	0.7481
Model	2	7.15	3.58	64.52	< 0.0001	
Residual	17	0.9422	0.0554			
Lack of Fit	12	0.8111	0.0676	2.58	0.1523	
Pure Error	5	0.1311	0.0262			
Total	19	8.09				

Total Alpha-Galactosides (mg/g dry bean) (R^2=0.8144, R^2 Adj=0.7796, CV=45.77%)						
Intercept						6.30
A-Time	1	1332.25	1332.25	44.11	< 0.0001	-11.54
B-Temp	1	136.62	136.62	4.52	0.0493	-3.70
A^2	1	651.23	651.23	21.56	0.0003	11.41
Model	3	2120.10	706.70	23.40	< 0.0001	
Residual	16	483.27	30.20			
Lack of Fit	11	391.98	35.63	1.95	0.2382	
Pure Error	5	91.29	18.26			
Total	19	2603.37				

*df = degree of freedom.
**R^2 Adj = Adjusted R^2.
#CV=Coefficient of Variation.

The raffinose content from germinated beans ranged from 0.87 to 8.35 mg/g dry bean. The significant terms contributing to the predictive model are the linear effects of time and temperature, the interactive term of time and temperature, and the quadratic term of time. The model was overall significant with a p-value of <0.0001, and an R^2 of 0.8462. Lack of fit was found to be not significant, with a p-value of 0.7099, indicating that this model for raffinose was statistically sound. The predictive model for raffinose content using the coded factor levels was:

$$Raffinose = 2.06 - 1.86 \times Time + 0.6609 \times Temp - 0.8513 \times Time * Temp + 1.88 \times Time^2 \quad \textbf{(9)}$$

Looking at the coefficient estimates, the quadratic time term has the largest effect on raffinose content, followed by the linear time term, the time*temp interaction term, and finally the temperature term. The linear coefficient estimates are interpreted to mean that raffinose content goes down as both the time goes up, and as the temperature of germination goes down. However, Figure 4.3 shows us that the raffinose content at a high temperature and high time is lower than the raffinose content obtained at a low temperature and high time. This is the influence of the

significant negative time*temp term, which indicates that the interaction of the germination time and temperature will cause the raffinose content to be lower than what is expected. The quadratic time coefficient had the greatest effect on raffinose content, and the positive value implies that there is a point of minimum for the raffinose content response, that is, as duration of germination increases, raffinose content decreases until the minimum point and then starts to increase (Figure 4.3). The high raffinose content at high temperature suggests the breakdown of verbascose into raffinose and galactose sugars.

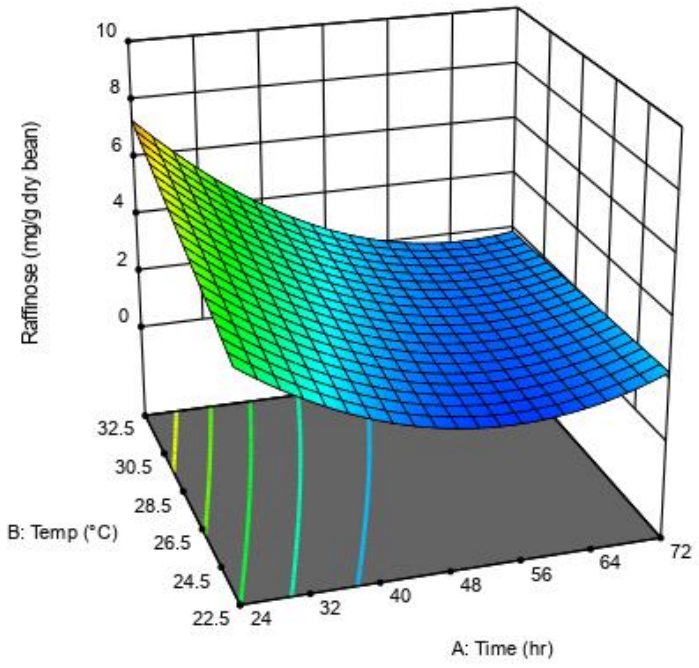


Figure 4.3 Response surface profile of the effect of time (hr) and temperature (°C) on the raffinose content of germinated Dynasty beans, given that light exposure is 12 hours.

The obtained results are contrary to published literature indicating that generally, raffinose content goes down during germination. As seen before, the variance in raffinose content within the batch

of seeds could be large enough that different 150g aliquots of beans would have significantly different levels of raffinose. This has yet to be experimentally determined.

The stachyose content in germinated beans ranged from 0.38 to 35.85 mg/g dry bean. A similar predictive model to raffinose was obtained, with significant terms being the linear time, linear temperature, the bilinear time*temp, and the quadratic time terms. The model was significant with a p-value of <0.0001 and a non-significant lack of fit (p-value=0.2074). The R^2 value was 0.8479, suggesting a good fit of the model to the data (Table 4.4). The predictive model for stachyose content using the coded factor levels was:

$$Stachyose = 3.61 - 9.02 \times Time - 4.29 \times Temp + 4.24 \times Time * Temp + 8.78 \times Time^2 \quad \textbf{(10)}$$

While the significant terms in the raffinose and stachyose model were the same, their effects on the sugar content were completely different. The linear time coefficient estimate was the most influential on stachyose content, its negative value indicating that as duration of germination increases, stachyose content decreases. The linear temperature coefficient estimate was also influential on stachyose content, but unlike for raffinose content, the term here is negative. As indicated by the negative coefficient estimates, when both temperature of germination and the duration of germination increases, the stachyose content of the bean decreases. Despite this trend, in Figure 4.4 it can be observed that the stachyose content at high temperature and high time is higher than the stachyose content at high temperature and low time. This is explained by the significant negative bilinear time*temp interaction term, which indicates that the interaction between the time and temperature of germination causes the stachyose content to be higher than what it is expected to be. In addition, the decrease in stachyose was much larger than the decreases

in the other alpha-galactosides. This is due to alpha-galactosidase, which attacks stachyose first before the other alpha-galactosides (Dey 1985).

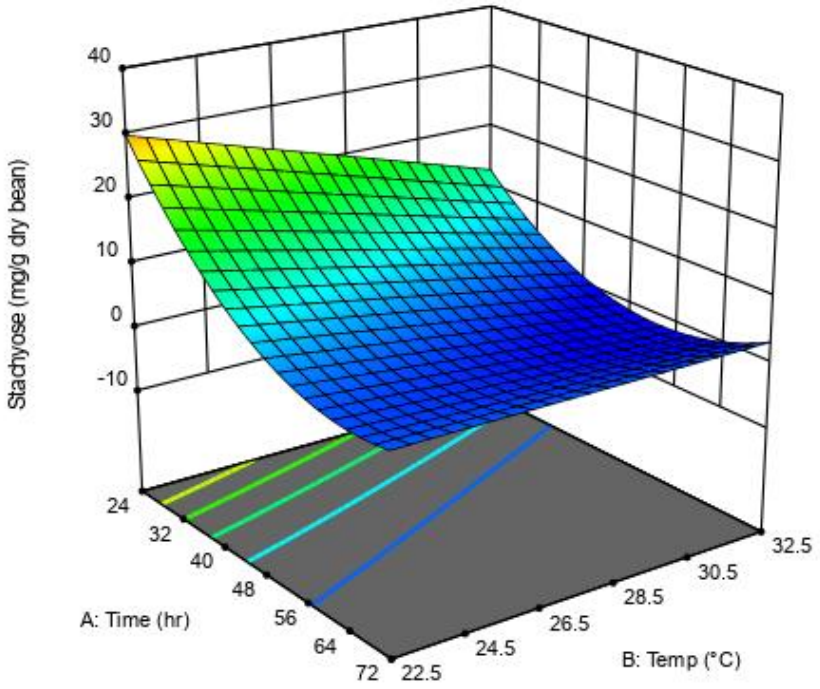


Figure 4.4 Response surface profile of the effect of time (hr) and temperature (°C) on the stachyose content of germinated Dynasty beans, given that light exposure is 12 hours.

The verbascose content in germinated beans ranged from 0.39 to 2.38 mg/g dry bean. The model was found to be significant with a p-value of <0.0001 and lack of fit was not significant, with a p-value of 0.1523, indicating a statistically sound model. The R^2 was 0.8836, suggesting that the model fit the data well. The predictive model for verbascose content using the coded factor levels was:

$$Verbascose = 0.6337 - 0.6598 \times Time + 0.7481 \times Time^2 \quad \textbf{(11)}$$

Only the linear time term and the quadratic time term was found to have a significant effect on the verbascose content, and the quadratic term had the higher influence than the linear term. This quadratic term can be found visually in Figure 4.5, where the verbascose content decreases to a minimum, before increasing.

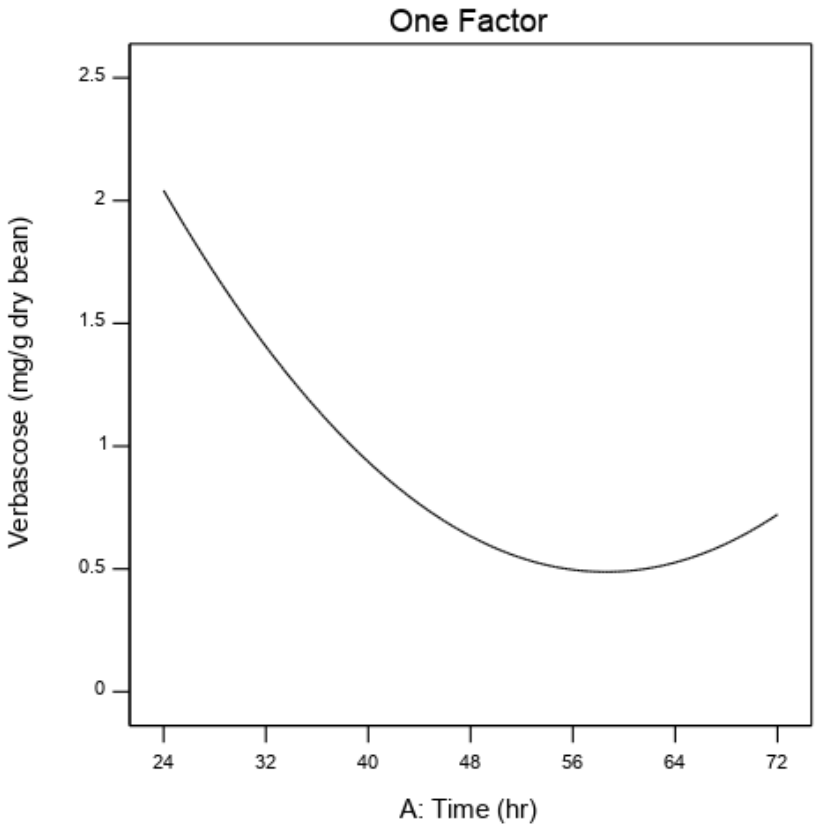


Figure 4.5 One factor analysis of the effect of time (hr) on verbascose content of germinated Dynasty beans, given that light exposure is 12 hours and temperature is 27.5°C.

It was thought that temperature would have a significant effect on verbascose content, like raffinose and stachyose. However, since alpha-galactosidase attacks stachyose first, before raffinose and then verbascose, the increased temperature would have a smaller to no effect on verbascose than on raffinose or stachyose.

The model for total alpha-galactosides has an R^2 of 0.8144, with a significant model (<0.0001) and a non-significant lack of fit (0.2382). The predictive model for total alpha-galactoside content using the coded factor levels was:

$$Total\ Alpha\ Galactosides = 6.30 - 11.54 \times Time - 3.70 \times Temp + 11.41 \times Time^2 \quad \textbf{(12)}$$

The terms that significantly affect the model are both linear terms for time and temperature, and the quadratic time term.

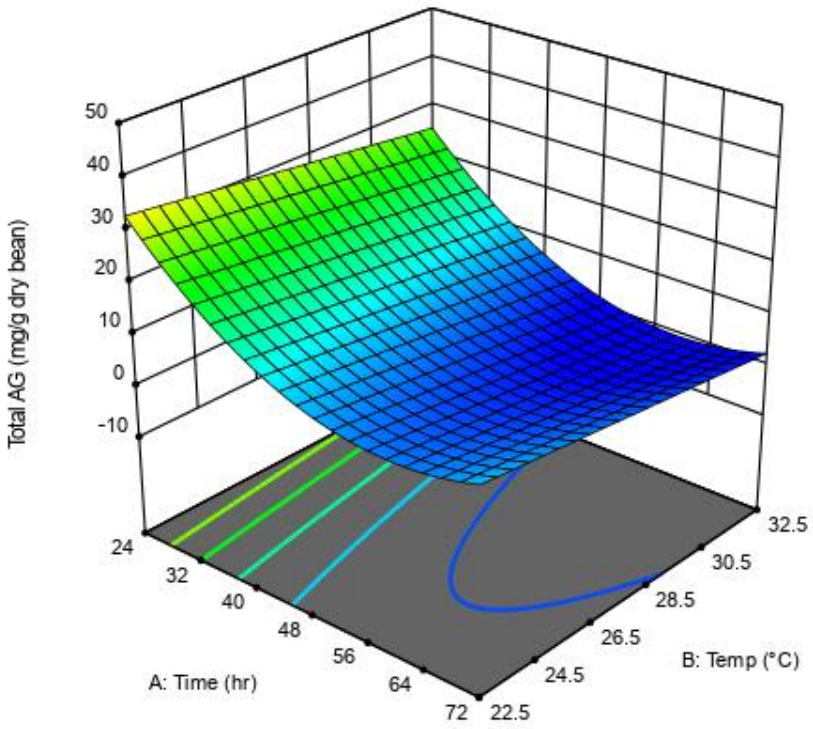


Figure 4.6 Response surface profile of the effect of time (hr) and temperature (°C) on the total alpha-galactoside content of germinated Dynasty beans, given that light exposure is 12 hours.

Looking at Figure 4.6, it is clear that time plays a bigger role in the reduction of total alpha-galactosides than temperature. This is consistent with past literature where researchers found that

longer germination time translated into higher reduction of total alpha-galactosides (Oboh et al. 2000; Jaya and Venkataraman 1981; Vidal-Valverde et al. 2002a).

4.2.2.2 Other soluble sugars

When seeds are germinated, enzymes within the seed are activated and cleave alpha-galactosides into their constituent sugars: glucose, fructose, sucrose and galactose. Table 4.5 summarizes the sucrose, glucose, galactose and fructose contents in the 20 trials.

Table 4.5 Experimental Data of sugar content for the three variable, 3 level CCD analysis.

Trial	Factors*			Responses			
	X_1	X_2	X_3	Sucrose (mg/g dry bean)	Glucose (mg/g dry bean)	Galactose (mg/g dry bean)	Fructose (mg/g dry bean)
1	-1	-1	-1	29.44**±0.59^{abc}	0.20±0.03^{c}	0.52±0.00^{ab}	0.36±0.01^{bc}
2	1	-1	-1	40.62±0.88^{abc}	1.01±0.03^{ab}	0.28±0.01^{abcd}	1.04±0.07^{ab}
3	-1	1	-1	32.82±0.26^{abc}	0.25±0.01^{bc}	0.50±0.03^{abc}	0.33±0.02^{bc}
4	1	1	-1	43.45±0.98^{ab}	0.95±0.01^{abc}	0.17±0.01^{d}	0.78±0.01^{abc}
5	-1	-1	1	24.98±0.65^{bc}	0.28±0.01^{bc}	0.46±0.01^{abcd}	0.19±0.01^{c}
6	1	-1	1	52.39±0.43^{ab}	0.54±0.00^{abc}	0.27±0.01^{abcd}	0.59±0.02^{abc}
7	-1	1	1	13.76±0.19^{c}	0.51±0.00^{abc}	0.57±0.02^{a}	0.28±0.01^{bc}
8	1	1	1	58.36±1.27^{a}	0.77±0.02^{abc}	0.36±0.01^{abcd}	0.42±0.01^{bc}
9	-1	0	0	38.56±0.72^{abc}	0.25±0.02^{bc}	0.31±0.03^{abcd}	0.43±0.01^{abc}
10	1	0	0	42.81±0.54^{abc}	0.97±0.05^{abc}	0.16±0.01^{d}	0.90±0.02^{abc}
11	0	-1	0	39.22±1.41^{abc}	0.61±0.01^{abc}	0.31±0.01^{abcd}	0.72±0.03^{abc}
12	0	1	0	56.19±0.17^{a}	0.67±0.01^{abc}	0.36±0.01^{abcd}	0.56±0.03^{abc}
13	0	0	-1	36.33±2.01^{abc}	1.11±0.02^{a}	0.35±0.01^{abcd}	1.18±0.08^{a}
14	0	0	1	42.94±0.95^{abc}	0.66±0.01^{abc}	0.22±0.01^{bcd}	0.77±0.00^{abc}
15	0	0	0	54.14±3.82^{ab}	0.38±0.01^{abc}	0.37±0.02^{abcd}	0.57±0.03^{abc}
16	0	0	0	44.71±1.41^{ab}	0.89±0.03^{abc}	0.18±0.01^{d}	0.87±0.05^{abc}
17	0	0	0	52.74±1.43^{ab}	0.77±0.01^{abc}	0.17±0.00^{d}	0.75±0.00^{abc}
18	0	0	0	43.25±0.32^{abc}	0.59±0.01^{abc}	0.18±0.01^{d}	0.63±0.05^{abc}
19	0	0	0	50.18±0.75^{ab}	0.72±0.12^{abc}	0.31±0.01^{abcd}	0.51±0.01^{abc}
20	0	0	0	55.02±1.71^{a}	0.83±0.01^{abc}	0.18±0.01^{d}	0.53±0.02^{abc}

*X_1=Time, X_2=Temperature, X_3=Light.
**Means are recorded as average ± standard deviation, and N=3. Means with the same letter in a column are not significantly different.

Like in the previous section, RSM was used to generate the predictive model for each sugar, but only the significant terms were included in the ANOVA analysis. Unlike the models for alpha-

galactosides, the models for each of the soluble sugars exhibited lower R^2 values, which indicates that these responses are not as well fit by the models. The ANOVA analyses for each soluble sugar are listed in Table 4.6.

Table 4.6 ANOVA for the CCD analyses for soluble sugars, with significant terms included.

Source	df[*]	Sum of Squares	Mean Square	F-value	p-value	Coefficient Estimate
Sucrose (mg/g dry bean) (R^2=0.5833, R^2 Adj[**]=0.5343, CV[#]=18.25%)						
Intercept						47.47
A-Time	1	962.06	962.06	15.93	0.0009	9.81
A^2	1	475.60	475.60	7.87	0.0122	-9.75
Model	2	1437.67	718.83	11.90	0.0006	
Residual	17	1026.87	60.40			
Lack of Fit	12	903.48	75.29	3.05	0.1134	
Pure Error	5	123.39	24.68			
Total	19	2464.53				
Glucose (mg/g dry bean) (R^2=0.5253, R^2 Adj=0.4990, CV=29.91%)						
Intercept						0.6481
A-Time	1	0.7487	0.7487	19.92	0.0003	0.2736
Model	1	0.7487	0.7487	19.92	0.0003	
Residual	18	0.6765	0.0376			
Lack of Fit	13	0.5004	0.0385	1.09	0.4988	
Pure Error	5	0.1761	0.0352			
Total	19	1.43				
Galactose (mg/g dry bean) (R^2=0.5725, R^2 Adj=0.5222, CV=28.24%)						
Intercept						0.2620
A-Time	1	0.1275	0.1275	16.51	0.0008	-0.1129
A^2	1	0.0483	0.0483	6.26	0.0229	0.0983
Model	2	0.1758	0.0879	11.38	0.0007	
Residual	17	0.1313	0.0077			
Lack of Fit	12	0.0945	0.0079	1.07	0.5066	
Pure Error	5	0.0368	0.0074			
Total	19	0.3071				

Fructose (mg/g dry bean) (R^2=0.6506, R^2 Adj=0.5851, CV=26.84%)						
Intercept						0.7095
A-Time	1	0.4583	0.4583	16.56	0.0009	0.2141
C-Light	1	0.2051	0.2051	7.41	0.0151	-0.1432
A^2	1	0.1610	0.1610	5.82	0.0282	-0.1794
Model	3	0.8244	0.2748	9.93	0.0006	
Residual	16	0.4428	0.0277			
Lack of Fit	11	0.3417	0.0311	1.54	0.3330	
Pure Error	5	0.1011	0.0202			
Total	19	1.27				

*df = degree of freedom.
**R^2 Adj = Adjusted R^2.
#CV=Coefficient of Variation.

Sucrose content in germinated beans ranged from 13.76 to 58.36 mg/g dry bean. The model was found significant (p-value=0.0006), lack of fit was found to be non-significant (p-value=0.1134), and the R^2 was 0.5833, all indicating an adequate model. The predictive model for sucrose content using the coded factor levels was:

$$Sucrose = 47.47 + 9.81 \times Time - 9.75 \times Time^2 \quad \textbf{(13)}$$

Only two terms were found to have a significant effect on sucrose content: the linear time term, which held the greater effect, and the quadratic time term. While the linear time term was positive, indicating that as the length of germination increases, so does the sucrose content, the quadratic time term was negative, indicating that sucrose content reaches a maximum before starting to decrease (Figure 4.7).

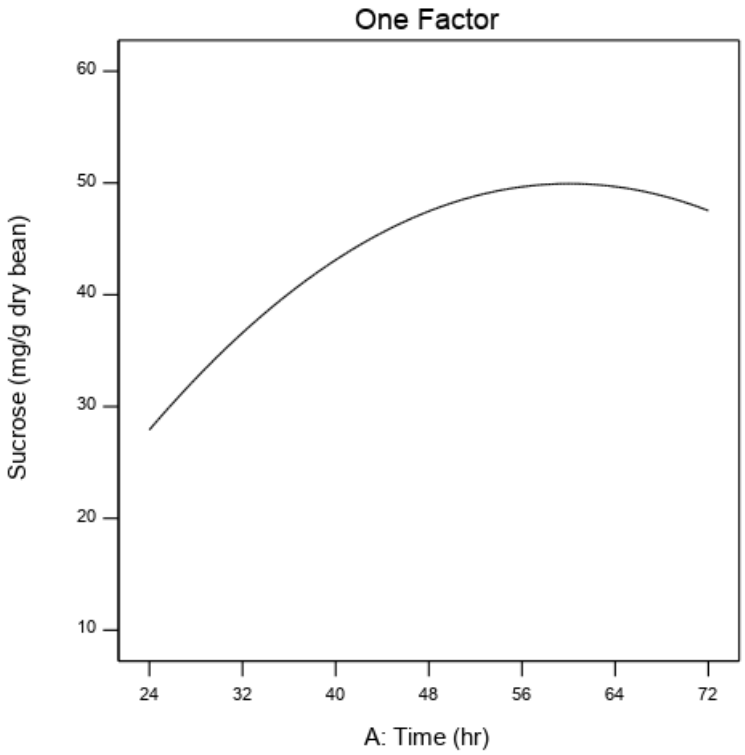


Figure 4.7 One factor analysis of the effect of time (hr) on sucrose content of germinated Dynasty beans, given that light exposure is 12 hours and temperature is 27.5°C.

Sucrose content was found to increase over time, presumably due to the work of alpha-galactosidase, which hydrolyzes alpha-galactosides into smaller sugars (Martínez-Villaluenga, Frias, and Vidal-Valverde 2008). The sucrose content results are in accordance with a previous study by Wang et al. (1997), who found both the linear and the quadratic time term to significantly affect the sucrose content. They also found that germination temperature exhibited a significant positive effect on sucrose content, that is, sucrose content increased as germination temperature increased.

Glucose content in germinated beans ranged from 0.20 to 1.11 mg/g dry bean. The model for glucose was found to be significant (p-value=0.0003) with a non-significant lack of fit (p-value=0.4988), and an R^2 of 0.5253. The predictive model for glucose content using the coded factor levels was:

$$Glucose = 0.6481 + 0.2736 \times Time \quad \textbf{(14)}$$

The only term that was found to significantly affect the glucose content was the linear time term. The linear time term has a positive value, indicating that as duration of germination increases, so does glucose content (Figure 4.8). This trend is consistent with prior research (Amuti and Pollard 1977). It is surprising to see that there was no quadratic time effect with glucose content, compared all the other sugars.

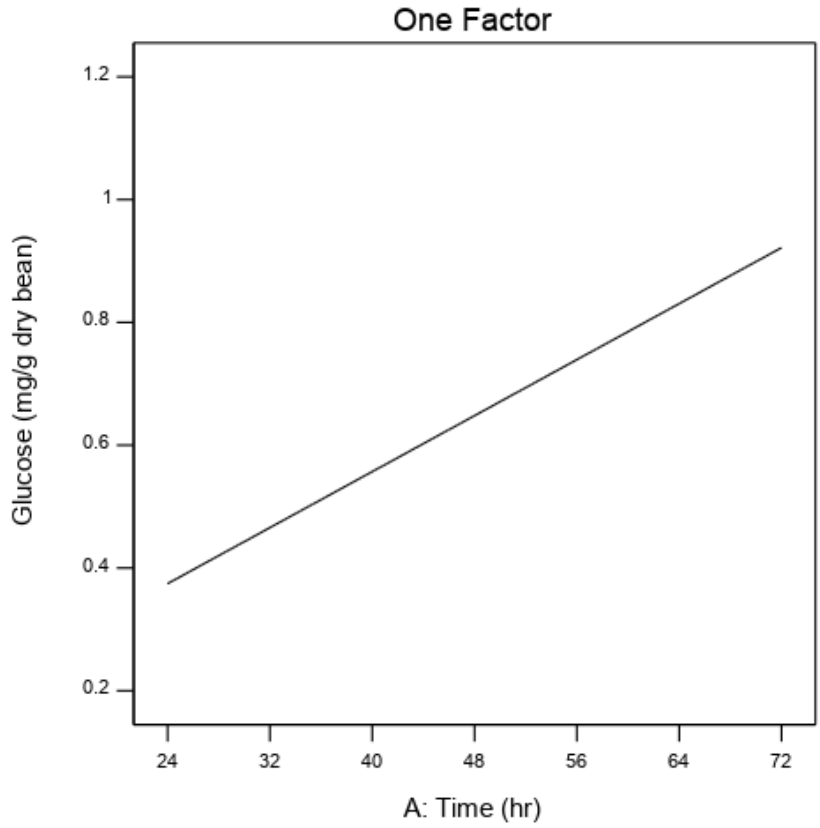


Figure 4.8 One factor analysis of the effect of time (hr) on glucose content of germinated Dynasty beans, given that light exposure is 12 hours and temperature is 27.5°C.

Galactose content ranged from 0.17 to 0.57 mg/g dry bean. The model for galactose was found to be significant (p-value=0.0007) with an insignificant lack of fit (p-value=0.5066), as well as R^2 value 0f 0.5725, suggesting an okay fit of the model. The predictive model for galactose content using the coded factor levels was:

$$Galactose = 0.2620 - 0.1129 \times Time + 0.0983 \times Time^2 \quad \textbf{(15)}$$

Like with sucrose and verbascose, only the linear time and the quadratic time term had a significant effect on galactose content. Unlike sucrose, the negative linear time term indicated that as duration of germination went up, the galactose content went down, and the positive quadratic time term indicated that galactose content decreased to a minimum before increasing (Figure 4.9).

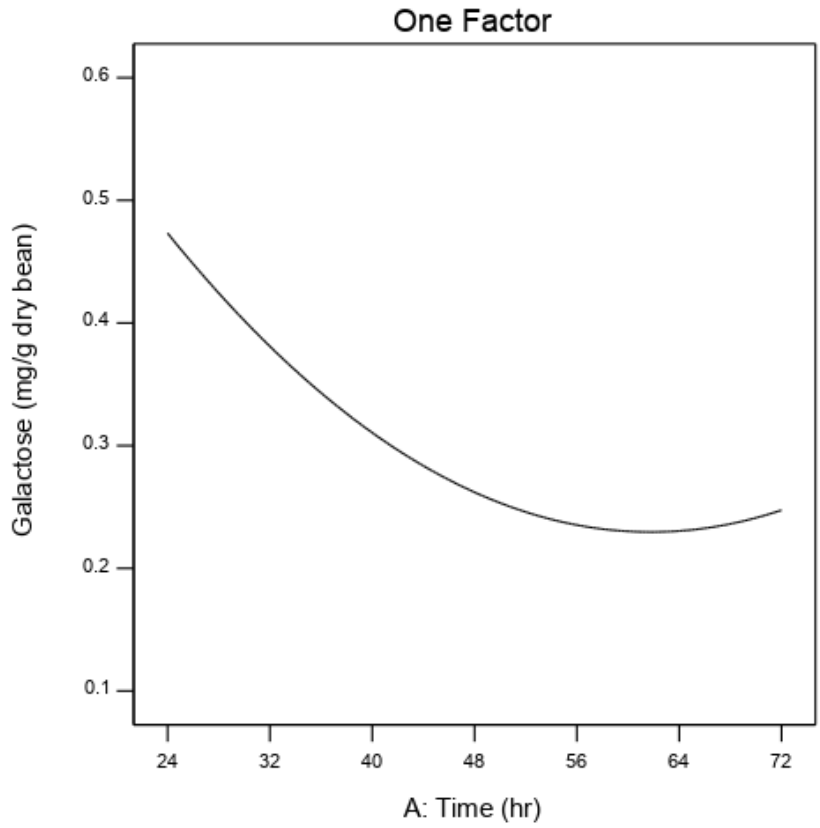


Figure 4.9 One factor analysis of the effect of time (hr) on galactose content of germinated Dynasty beans, given that light exposure is 12 hours and temperature is 27.5°C.

Fructose content in germinated beans ranged from 0.19 to 1.18 mg/g dry bean. The fructose content was adequately predicted by the model, with a non-significant lack of fit (p-value=0.3330) and an R^2 of 0.6506. The predictive model for fructose content using the coded factor levels was:

$$Fructose = 0.7095 + 0.2141 \times Time - 0.1432 \times Light - 0.1794 \times Time^2 \quad \textbf{(16)}$$

Like with the other sugars, both the linear time term and the quadratic time term had a significant effect on fructose content, with the linear time term contributing the largest effect. In addition, this was the only sugar that was affected by light exposure, as shown by the significant negative linear light term, which indicates that as light exposure increases, fructose content decreases during germination (Figure 4.10).

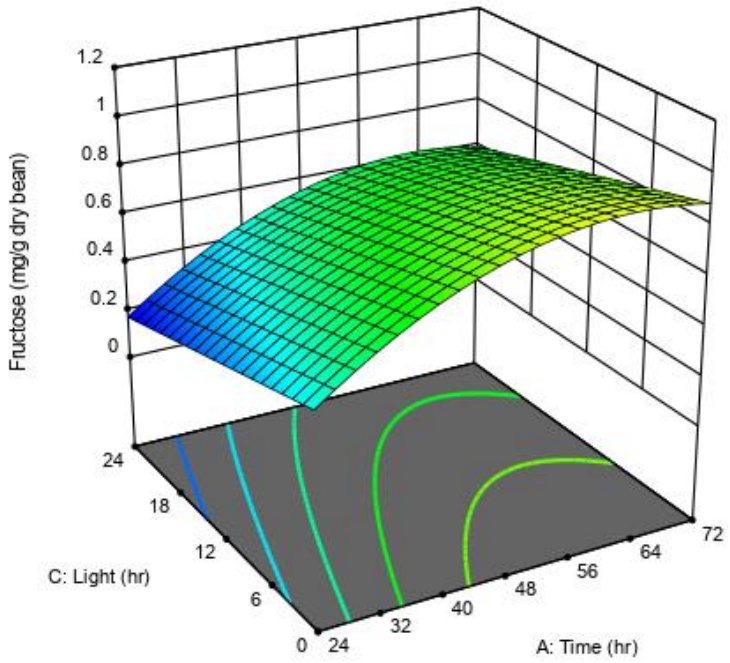


Figure 4.10 Response surface profile of the effect of time (hr) and light (hr) on the fructose content of Dynasty beans, given that temperature is 27.5°C.

Research by Amuti and Pollard (1977) indicates that fructose content increased in germinating bambara groundnut, but only after 7 days of germination. Kaczmarska et al. (2017) found that fructose content increased significantly when both lupin seeds and soybeans were germinated, and that fructose content in soybeans germinated under 0 hours of light was significantly higher than soybeans germinated under 24 hours of light.

4.2.3 Protein and Protein Digestibility

When comparing the raw and soaked Dynasty beans, soaking did not significantly affect the protein content, or the protein digestibility of the beans (Figure 4.11, Figure 4.12).

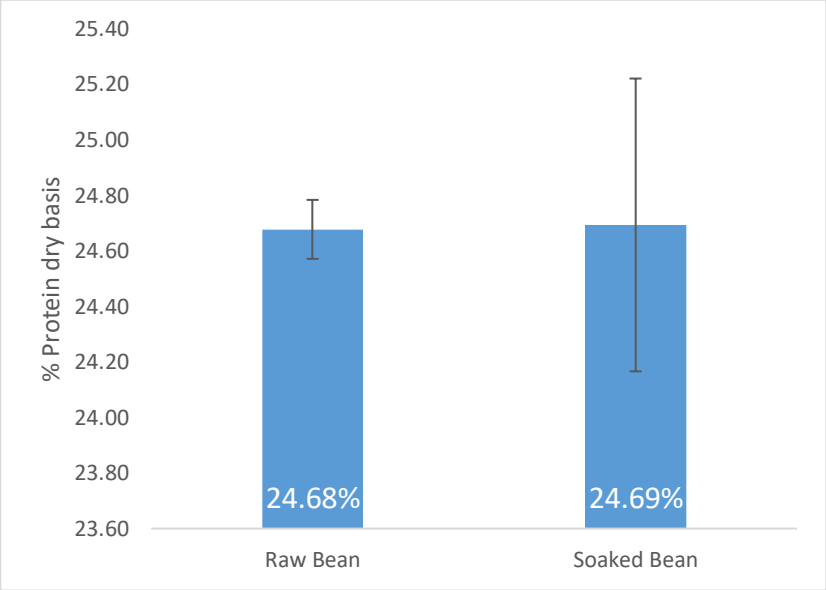


Figure 4.11 Protein content in raw and soaked Dynasty beans

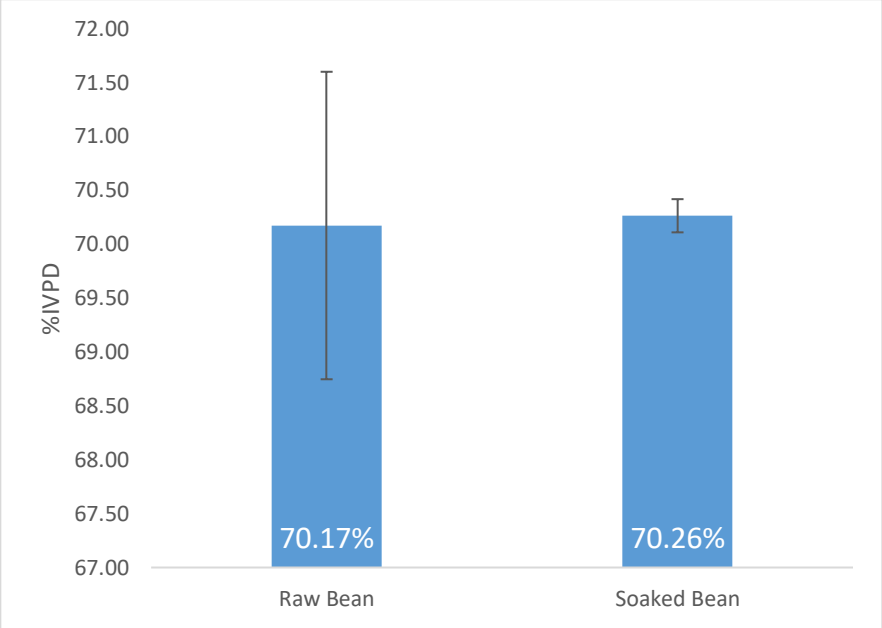


Figure 4.12 *In-Vitro* Protein Digestibility in raw and soaked Dynasty beans

Table 4.7 summarizes the protein content and *in-vitro* protein digestibility of the 20 trials. Most protein values and IVPD values were not significantly different from each other.

Table 4.7 Experimental data of protein content and IVPD for the three variable, 3 level CCD analysis.

Trial	Factors*			Responses	
	X_1	X_2	X_3	**Protein Content (% dry basis)**	**IVPD (%)**
1	-1	-1	-1	25.24**±0.28^{ab}	71.60±0.20^{ab}
2	1	-1	-1	25.92±0.24^{ab}	70.84±0.13^{b}
3	-1	1	-1	25.19±0.05^{ab}	71.63±0.51^{ab}
4	1	1	-1	26.13±0.18^{a}	70.56±0.02^{b}
5	-1	-1	1	24.95±0.13^{b}	72.52±0.15^{a}
6	1	-1	1	24.94±0.15^{b}	71.34±0.01^{ab}
7	-1	1	1	25.47±0.19^{ab}	71.72±0.14^{ab}
8	1	1	1	25.96±0.01^{ab}	71.05±0.04^{ab}
9	-1	0	0	25.49±0.01^{ab}	71.16±0.70^{ab}
10	1	0	0	25.88±0.17^{ab}	70.88±0.73^{ab}
11	0	-1	0	25.05±0.03^{ab}	70.47±0.07^{b}
12	0	1	0	25.66±0.11^{ab}	71.61±0.21^{ab}
13	0	0	-1	25.41±0.09^{ab}	70.30±0.09^{b}
14	0	0	1	25.59±0.13^{ab}	70.60±0.18^{b}
15	0	0	0	25.05±0.03^{ab}	71.27±0.30^{ab}
16	0	0	0	25.52±0.14^{ab}	70.96±0.20^{ab}
17	0	0	0	25.31±0.36^{ab}	70.85±0.43^{b}
18	0	0	0	25.59±0.16^{ab}	70.65±0.03^{b}
19	0	0	0	25.01±0.16^{ab}	71.05±0.11^{ab}
20	0	0	0	25.76±0.17^{ab}	70.64±0.37^{b}

*X_1=Time, X_2=Temperature, X_3=Light.

**Means are recorded as average ± standard deviation, and N=3. Means with the same letter in a column are not significantly different.

RSM was used to analyze the effect of the linear, interaction, and quadratic effects on the two responses, however only the significant parameters were used to generate the predictive model for each response. The ANOVA analyses of each predictive model are displayed in Table 4.8.

Table 4.8 ANOVA for the CCD analyses for protein content and IVPD, with significant terms included.

Source	df*	Sum of Squares	Mean Square	F-value	p-value	Coefficient Estimate
Protein (% dry basis) (R^2=0.4669, R^2 Adj**=0.4042, CV#=1.09%)						
Intercept						25.46
A-Time	1	0.6128	0.6128	7.96	0.0118	0.2475
B-Temp	1	0.5330	0.5330	6.93	0.0175	0.2309
Model	2	1.15	0.5729	7.44	0.0048	
Residual	17	1.31	0.0770			
Lack of Fit	12	0.8588	0.0716	0.7962	0.6564	
Pure Error	5	0.4495	0.0899			
Total	19	2.45				
IVPD (%) (R^2=0.2865, R^2 Adj=0.2489, CV=0.66%)						
Intercept						3.61
A-Time	1	1.57	1.57	7.23	0.0150	-9.02
Model	1	1.57	1.57	7.23	0.0150	
Residual	18	3.90	0.2169			
Lack of Fit	13	3.61	0.2776	4.70	0.0491	
Pure Error	5	0.2955	0.0591			
Total	19	5.47				

*df = degree of freedom.
**R^2 Adj = Adjusted R^2.
#CV=Coefficient of Variation.

The model for protein has an adequate R^2 value (0.4669), a significant model (p-value=0.0048) and a non-significant lack of fit (p-value=0.6564), indicating a statistically sound, but somewhat inadequate model. The predictive model for protein content using the coded factor levels was:

$$Protein = 25.46 - 0.2475 \times Time + 0.2309 \times Temp \quad \textbf{(17)}$$

The two terms that significantly affected the protein content were the linear time and linear temperature coefficient estimates, with time having the greater effect than temperature. Both terms were positive, indicating that as length of germination and ambient temperature went up, so did the protein content during germination (Figure 4.13). This is consistent with prior research, which

showed that germination increased protein contents in mung bean, cowpea, soybean, and chickpea (Dogra, Dhaliwal, and Kalia 2001; Khader 1983; Uppal and Bains 2012).

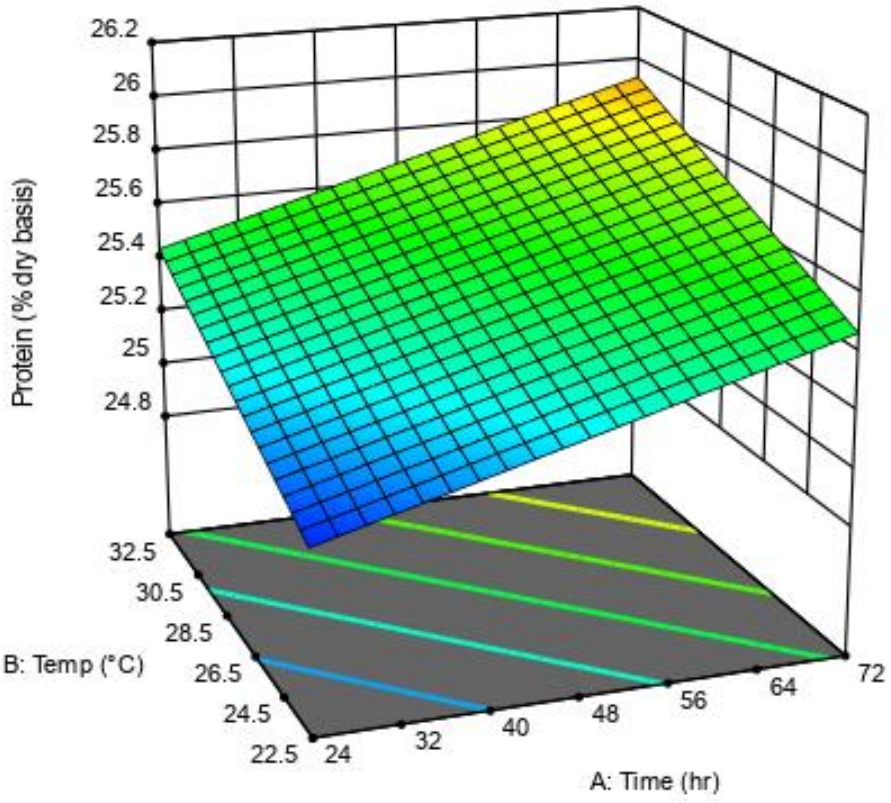


Figure 4.13 Response surface profile of the effect of time (hr) and temperature (°C) on the protein content of Dynasty beans, given that light exposure is 12 hours.

The germinated beans ranged from Despite the significant model (p-value=0.0150) and a non-significant lack of fit (p-value=0.0491) for IVPD, the R^2 value was 0.2865, suggesting that the model is inadequate to fit the data. The predictive model for IVPD using the coded factor levels was:

$$IVPD = 71.08 - 0.3960 \times Time \quad \textbf{(18)}$$

Only the linear time term affected the IVPD significantly, with the negative value indicating that as beans germinate for a longer period of time, the IVPD decreases (Figure 4.14Figure 4.12).

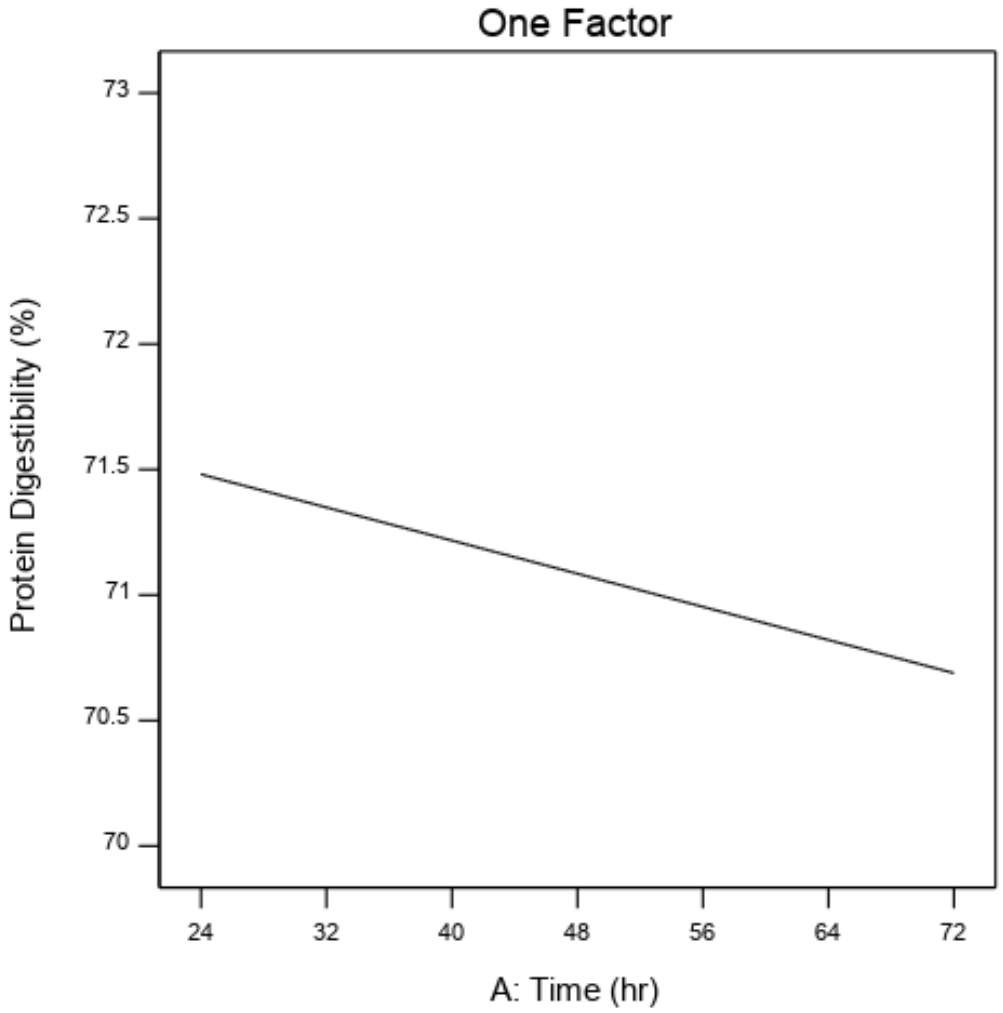


Figure 4.14 One factor analysis of the effect of time (hr) on *in-vitro* protein digestibility of germinated Dynasty beans, given that light exposure is 12 hours and temperature is 27.5°C.

This decrease in IVPD could be a result of the tannin content in the common bean. Tannins have been reported to bind with proteins and enzymes, which results in their inactivation. When beans are soaked and then germinated, the tannins leach out, enabling them to form these complexes with the protein and reduce IVPD (Elmaki, Babiker, and El Tinay 1999).

4.2.4 Resistant Starch

Looking at the raw and soaked Dynasty beans, soaking did not significantly affect the resistant starch content of the beans but did induce a loss in digestible starch (Figure 4.15). This suggests that the soaking treatment leached the digestible starch from the beans, however an analysis of the soaking water would be required to support this claim.

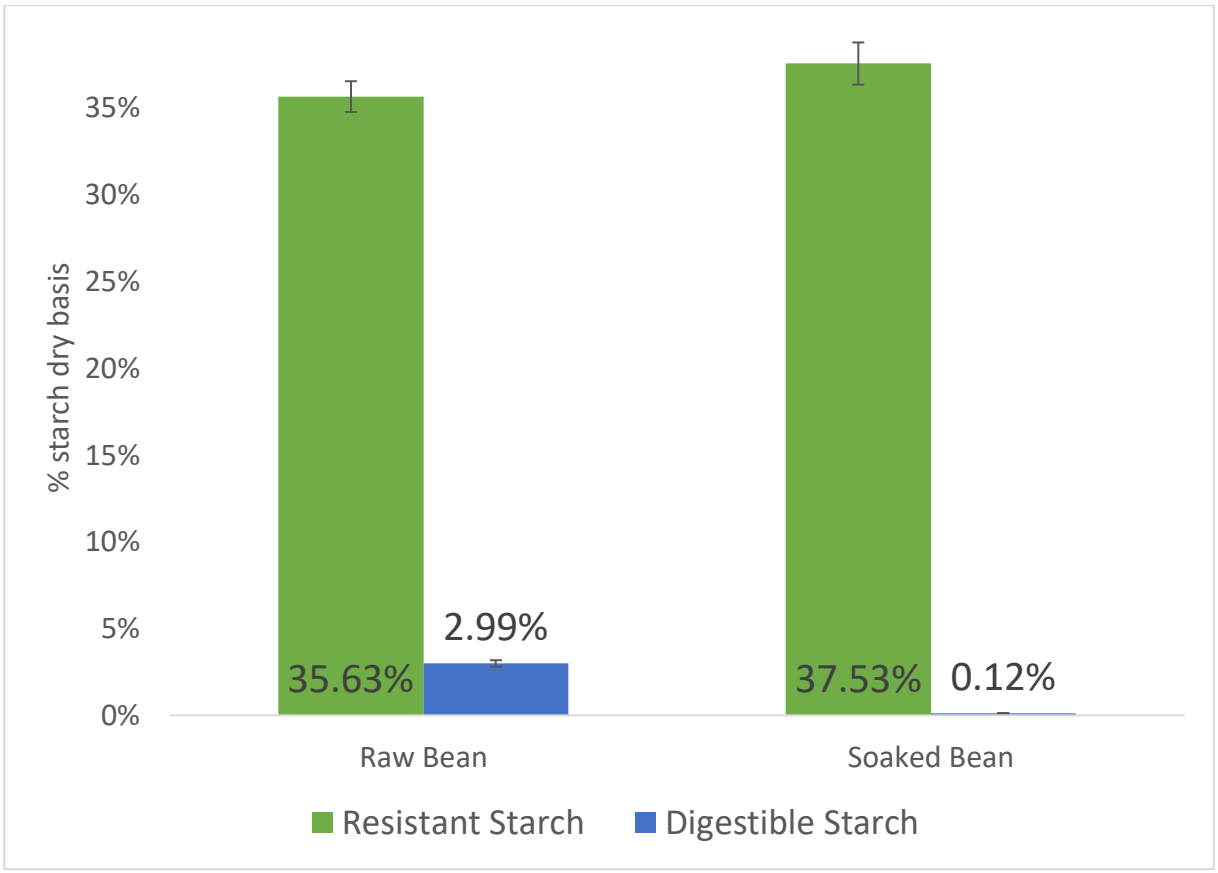


Figure 4.15 Resistant and digestible starch in raw and soaked Dynasty beans

Table 4.9 summarizes the RS content of the 20 trials. All values were not significantly different from each other, except for trials 2, 4, 6, 7, 9, 11 and 12.

Table 4.9 Experimental data of resistant starch content for the three variable, 3 level CCD analysis.

Trial	Factors*			Responses
	X_1	X_2	X_3	**Resistant Starch Content (% dry basis)**
1	-1	-1	-1	$35.66^{**}\pm0.98^{abc}$
2	1	-1	-1	37.97 ± 2.80^{a}
3	-1	1	-1	34.36 ± 0.87^{abc}
4	1	1	-1	39.55 ± 1.64^{a}
5	-1	-1	1	36.19 ± 0.43^{ab}
6	1	-1	1	36.79 ± 1.32^{a}
7	-1	1	1	37.27 ± 1.57^{a}
8	1	1	1	32.12 ± 2.40^{abc}
9	-1	0	0	38.25 ± 1.22^{a}
10	1	0	0	35.72 ± 0.53^{abc}
11	0	-1	0	37.48 ± 0.89^{a}
12	0	1	0	28.13 ± 1.70^{c}
13	0	0	-1	34.18 ± 1.14^{abc}
14	0	0	1	32.13 ± 1.60^{abc}
15	0	0	0	28.50 ± 0.84^{bc}
16	0	0	0	35.98 ± 1.10^{abc}
17	0	0	0	35.59 ± 1.15^{abc}
18	0	0	0	35.53 ± 2.11^{abc}
19	0	0	0	35.63 ± 1.06^{abc}
20	0	0	0	35.08 ± 1.01^{abc}

*X_1=Time, X_2=Temperature, X_3=Light.

**Means are recorded as average ± standard deviation, and N=4. Means with the same letter in a column are not significantly different.

RS was modeled by RSM however the model was found to be not significant (p-value=0.4875), with a significant lack of fit (p-value=0.4727). In addition, all terms were found to not have a significant effect on the resistant starch content. Table 4.10 displays the full ANOVA analysis.

Table 4.10 ANOVA for the CCD analyses for resistant starch, with all terms included

Source	df*	Sum of Squares	Mean Square	F-value	p-value	Coefficient Estimate
Resistant Starch (% dry basis) (R^2=0.4770, R^2 Adj**=0.0062, CV#=8.38%)						
Intercept						34.08
A-Time	1	0.0181	0.0181	0.0021	0.9645	0.0425
B-Temp	1	16.01	16.01	1.85	0.2038	-1.27
C-Light	1	5.20	5.20	0.6008	0.4562	-0.7213
AB	1	1.02	1.02	0.1183	0.7380	-0.3578
AC	1	18.13	18.13	2.09	0.1786	-1.51
BC	1	1.87	1.87	0.2158	0.6522	-0.4833
A^2	1	30.79	30.79	3.56	0.0887	3.35
B^2	1	1.88	1.88	0.2175	0.6509	-0.8276
C^2	1	0.6285	0.6285	0.0726	0.7931	-0.4781
Model	9	78.97	8.77	1.01	0.4875	
Residual	10	86.60	8.66			
Lack of Fit	5	44.69	8.94	1.07	0.4727	
Pure Error	5	41.90	8.38			
Total	19	165.57				

*df = degree of freedom.
**R^2 Adj = Adjusted R^2.
#CV=Coefficient of Variation.

One possible reason for the poor model could be a lack of good data. More work is needed to adequately investigate the modeling of RS content in germinated beans. Another reason could be the particle size of the flour, where although the flour was ground and sifted through a 500µm sieve several times, there could be a loss of resistant starch in the remaining fraction. A mill with a built in screen would have been a better choice for grinding the seeds into powder.

4.2.5 Total Phenolic Content

Comparing the raw and soaked Dynasty beans, soaking significantly decreased the TPC of the beans (Figure 4.16). However, it is important to note that the extraction method for TPC only extracted the free phenolics, not the bound phenolics.

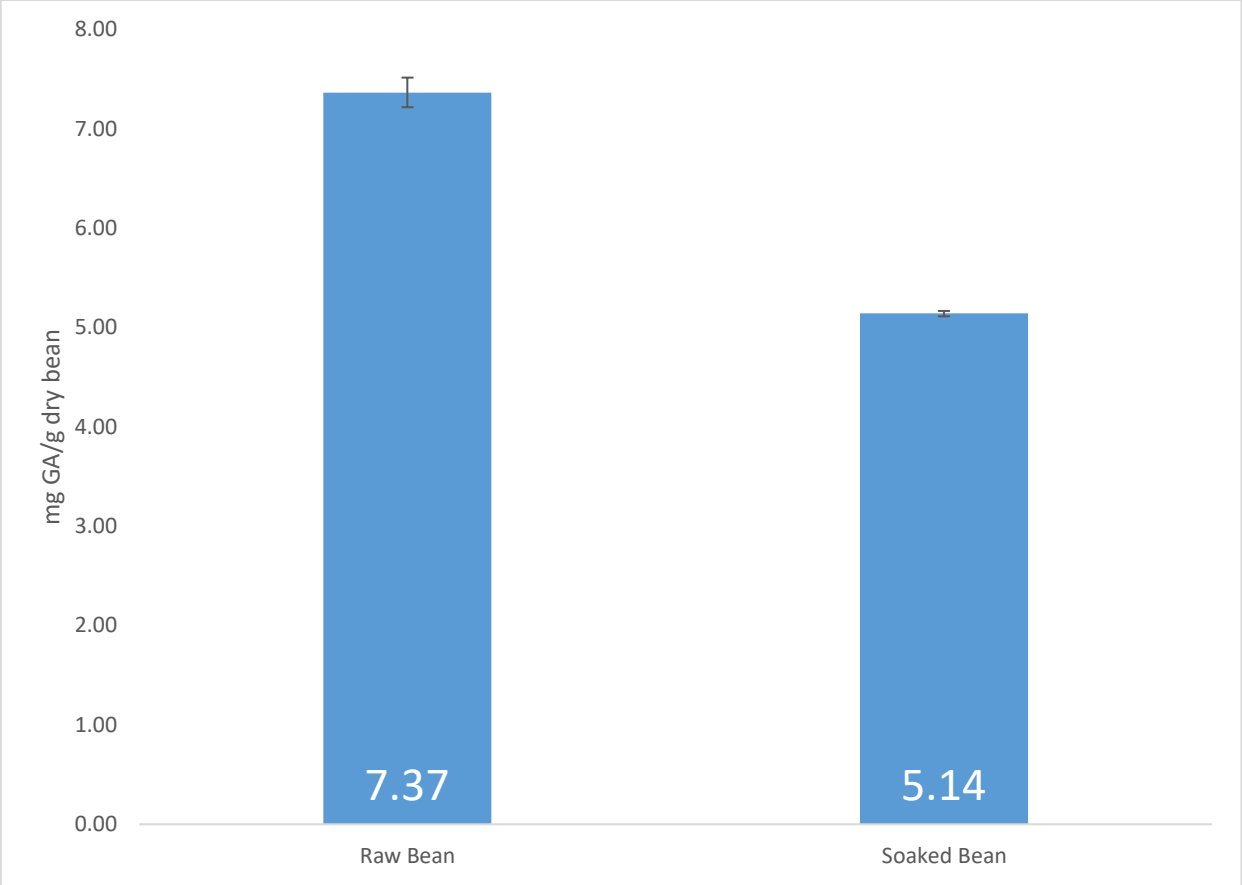


Figure 4.16 TPC in raw and soaked Dynasty beans

The total extractable phenolic content (TEPC) in the 20 trials is summarized in Table 4.11. TEPC content in germinated beans ranged from 4.23 to 5.70 mg GA/g dry bean. These values are slightly higher than previous research by Aguilera et al. (2014) and Saleh et al. (2017), which could be due to the different sources of beans.

Table 4.11 Experimental data of TEPC for the three variable, 3 level CCD analysis.

Trial	Factors*			Responses
	X_1	X_2	X_3	**TPC (mg GA/g bean dry basis)**
1	-1	-1	-1	$4.98^{**}\pm0.98^{ab}$
2	1	-1	-1	5.70 ± 0.04^{ab}
3	-1	1	-1	4.72 ± 0.16^{ab}
4	1	1	-1	5.60 ± 0.29^{a}
5	-1	-1	1	4.71 ± 0.16^{ab}
6	1	-1	1	4.74 ± 0.33^{ab}
7	-1	1	1	4.79 ± 0.14^{ab}
8	1	1	1	5.54 ± 0.29^{a}
9	-1	0	0	4.89 ± 0.13^{ab}
10	1	0	0	5.44 ± 0.20^{ab}
11	0	-1	0	5.42 ± 0.18^{ab}
12	0	1	0	5.45 ± 0.09^{ab}
13	0	0	-1	4.70 ± 0.14^{ab}
14	0	0	1	5.22 ± 0.13^{ab}
15	0	0	0	5.45 ± 0.07^{ab}
16	0	0	0	4.90 ± 0.06^{ab}
17	0	0	0	4.84 ± 0.18^{ab}
18	0	0	0	5.14 ± 0.19^{ab}
19	0	0	0	4.23 ± 0.14^{b}
20	0	0	0	5.08 ± 0.05^{ab}

*X_1=Time, X_2=Temperature, X_3=Light.

**Means are recorded as average ± standard deviation, and N=3. Means with the same letter in a column are not significantly different.

RSM was used to analyze the effect of the linear, interaction, and quadratic effects on TEPC, however only the significant parameters were used to generate the predictive model for each response. The ANOVA analyses of each predictive model are displayed in Table 4.12.

Table 4.12 ANOVA for the CCD analyses for TEPC, with significant terms included.

Source	df*	Sum of Squares	Mean Square	F-value	p-value	Coefficient Estimate
TPC (mg GA/g dry bean) (R^2=0.2995, R^2 Adj**=0.2606, CV#=6.60%)						
Intercept						5.08
A-Time	1	0.8646	0.8646	7.70	0.0125	0.2940
Model	1	0.8646	0.8646	7.70	0.0125	
Residual	18	2.02	0.1123			
Lack of Fit	13	1.19	0.0912	0.5452	0.8248	
Pure Error	5	0.8364	0.1673			
Total	19	2.89				

*df = degree of freedom.
**R^2 Adj = Adjusted R^2.
#CV=Coefficient of Variation.

Although the model is significant (p-value=0.0125), with a non-significant lack of fit (p-value=0.8248), the R^2 value is quite low (0.2995), suggesting that this model is inadequate. The linear time term was found to be the only coefficient estimate significantly affecting TEPC (Figure 4.17). The predictive model for TEPC using the coded factor levels was:

$$TEPC = 5.08 + 0.2940 \times Time \quad \textbf{(}19\textbf{)}$$

The absence of light as a significant effect is interesting, as Aguilera et al. (2014) found that beans germinated in the dark (0 hours of light) had significantly higher TPC than beans germinated in 12 hours of light.

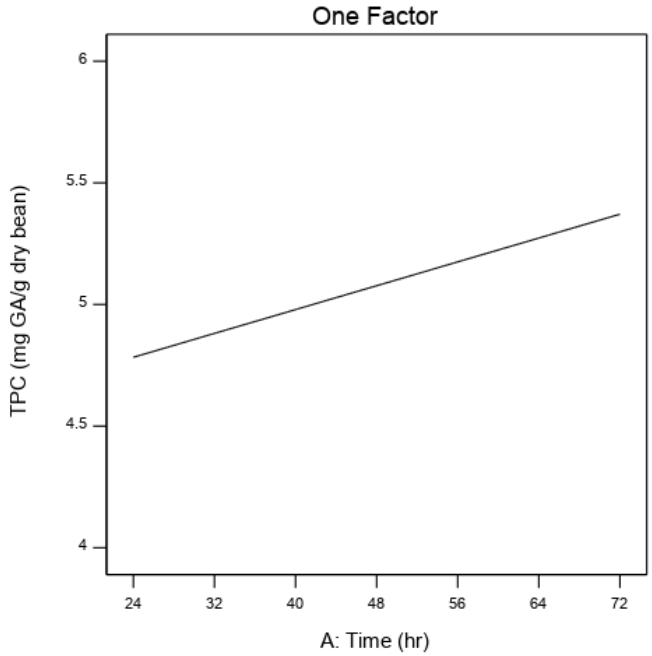


Figure 4.17 One factor analysis of the effect of time (hr) on TEPC of germinated Dynasty beans, given that light exposure is 12 hours and temperature is 27.5°C.

4.2.6 Antioxidant Capacity

Looking at the raw and soaked Dynasty beans, soaking significantly decreased both the DPPH and the ABTS antioxidant capacity of the beans (Figure 4.18).

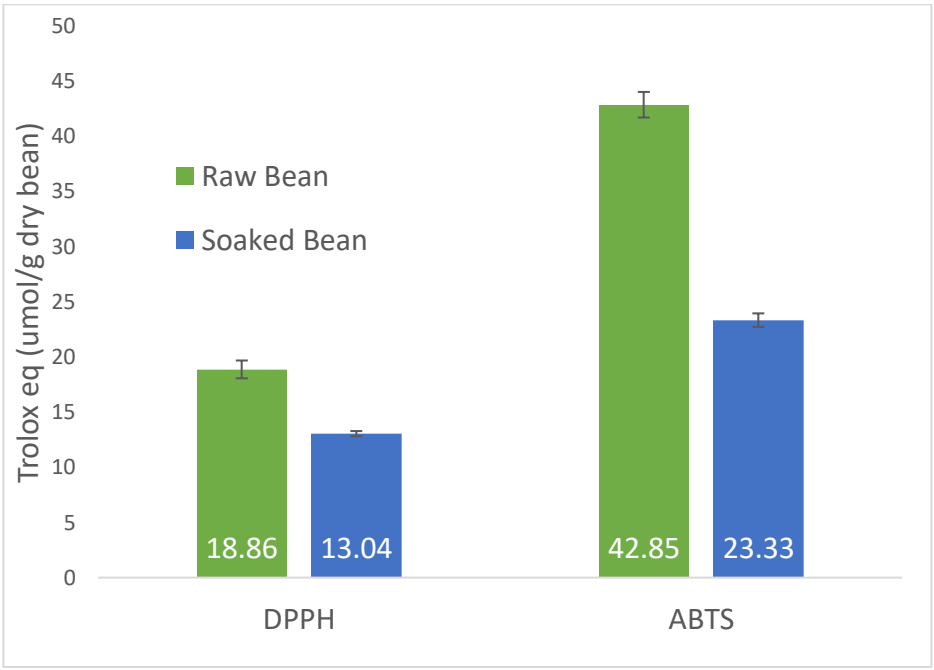


Figure 4.18 Antioxidant capacity in raw and soaked Dynasty beans

Table 4.13 summarizes the antioxidant capacity values obtained for the 20 trials.

Table 4.13 Experimental data of antioxidant capacity for the three variable, 3 level CCD analysis.

Trial	Factors*			Responses	
	X_1	X_2	X_3	**ABTS (µmol TE/g dry bean)**	**DPPH (µmol TE/g dry bean)**
1	-1	-1	-1	$26.24^{**}\pm1.26^{c}$	9.30 ± 0.19^{b}
2	1	-1	-1	34.32 ± 1.36^{abc}	11.10 ± 0.56^{ab}
3	-1	1	-1	30.58 ± 1.03^{bc}	9.49 ± 0.24^{b}
4	1	1	-1	42.22 ± 1.87^{a}	12.10 ± 0.41^{ab}
5	-1	-1	1	28.62 ± 1.28^{bc}	9.93 ± 0.25^{ab}
6	1	-1	1	29.20 ± 1.05^{bc}	11.59 ± 0.52^{ab}
7	-1	1	1	31.78 ± 1.44^{abc}	9.94 ± 0.30^{ab}
8	1	1	1	38.27 ± 1.52^{ab}	13.26 ± 0.21^{a}
9	-1	0	0	30.45 ± 1.27^{bc}	10.88 ± 0.38^{ab}
10	1	0	0	37.51 ± 0.57^{ab}	11.90 ± 0.29^{ab}
11	0	-1	0	32.95 ± 1.60^{abc}	9.92 ± 0.20^{b}
12	0	1	0	36.17 ± 1.37^{abc}	12.44 ± 0.45^{ab}
13	0	0	-1	31.22 ± 1.06^{abc}	9.48 ± 0.55^{b}
14	0	0	1	35.77 ± 0.45^{abc}	11.10 ± 0.41^{ab}
15	0	0	0	36.02 ± 0.75^{abc}	11.59 ± 0.18^{ab}
16	0	0	0	35.79 ± 0.71^{abc}	11.28 ± 0.38^{ab}
17	0	0	0	33.58 ± 0.52^{abc}	10.31 ± 0.13^{ab}
18	0	0	0	33.48 ± 0.66^{abc}	11.03 ± 0.25^{ab}
19	0	0	0	33.38 ± 0.54^{abc}	10.15 ± 0.45^{ab}
20	0	0	0	33.05 ± 0.33^{abc}	11.03 ± 0.34^{ab}

*X_1=Time, X_2=Temperature, X_3=Light.

**Means are recorded as average ± standard deviation, and N=3. Means with the same letter in a column are not significantly different.

RSM was used to analyze the effect of the linear, interaction, and quadratic effects on the two responses, however only the significant parameters were used to generate the predictive model for each response. The ANOVA analyses of each predictive model are displayed in Table 4.14.

Table 4.14 ANOVA for the CCD analyses for antioxidant capacity, with significant terms included.

Source	df*	Sum of Squares	Mean Square	F-value	p-value	Coefficient Estimate
ABTS (µmol TE/g dry bean) (R^2=0.7330, R^2 Adj**=0.7016, CV#=6.04%)						
Intercept						33.53
A-Time	1	114.56	114.56	27.96	< 0.0001	3.38
B-Temp	1	76.69	76.69	18.71	0.0005	2.77
Model	2	191.26	95.63	23.33	< 0.0001	
Residual	17	69.67	4.10			
Lack of Fit	12	60.93	5.08	2.90	0.1238	
Pure Error	5	8.74	1.75			
Cor Total	19	260.92				
DPPH (µmol TE/g dry bean) (%) (R^2=0.7143, R^2 Adj=0.6608, CV=5.74%)						
Intercept						10.89
A-Time	1	10.81	10.81	27.69	< 0.0001	1.04
B-Temp	1	2.90	2.90	7.44	0.0149	0.5389
C-Light	1	1.90	1.90	4.88	0.0422	0.4362
Model	3	15.61	5.20	13.34	0.0001	
Residual	16	6.24	0.3902			
Lack of Fit	11	4.68	0.4256	1.36	0.3869	
Pure Error	5	1.56	0.3124			
Cor Total	19	21.86				

*df = degree of freedom.
**R^2 Adj = Adjusted R^2.
#CV=Coefficient of Variation.

ABTS values ranged from 26.24 to 42.22 µmol TE/g dry bean. The model for ABTS was found to be significant (p-value<0.0001) with a non-significant lack of fit (p-value=0.1238). The R^2 value was 0.7330, suggesting an adequate, statistically sound fit. The predictive model for ABTS antioxidant capacity using the coded factor levels was:

$$ABTS = 33.53 + 3.38 \times Time + 2.77 \times Temp \quad \textbf{(20)}$$

The terms that had a significant effect on ABTS antioxidant capacity were both the linear time and temperature terms, with time having a greater effect than temperature. Both terms were positive,

indicating that as length of germination and ambient temperature increased, so did the antioxidant capacity of the beans (Figure 4.19).

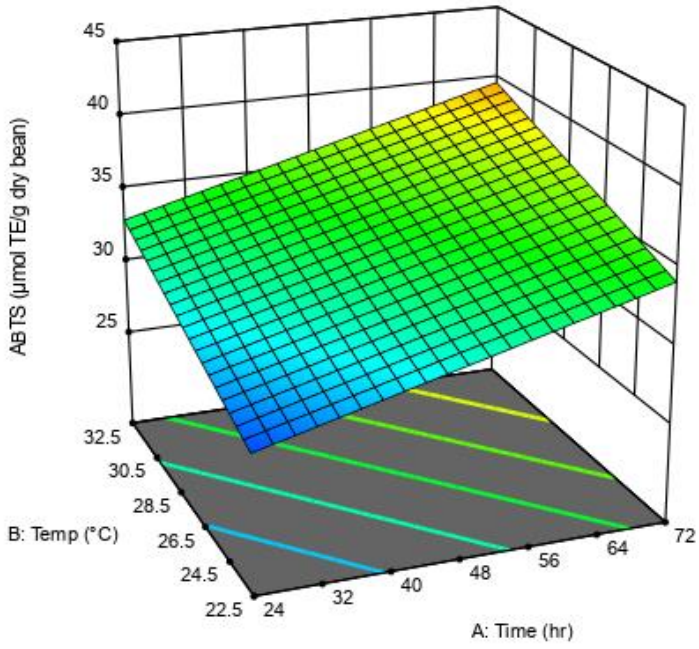


Figure 4.19 Response surface profile of the effect of time (hr) and temperature (°C) on the ABTS antioxidant capacity of Dynasty beans, given that light exposure is 12 hours.

DPPH values ranged from 9.30 to 13.26 µmol TE/g dry bean. The model for DPPH was also found to be significant (p-value=0.0001) with a non-significant lack of fit (p-value=0.3869). The R^2 value was 0.7143, suggesting an adequate, statistically sound fit. The terms that had a significant effect on DPPH antioxidant capacity were the linear time, temperature and light terms, with time having the greatest effect. All terms were positive, indicating that as length of germination, ambient temperature, and light exposure increased, so did the antioxidant capacity of the beans (Figure 4.20). The predictive model for DPPH using the coded factor levels was:

$$DPPH = 10.89 + 1.04 \times Time + 0.5389 \times Temp + 0.4362 \times Light \quad (21)$$

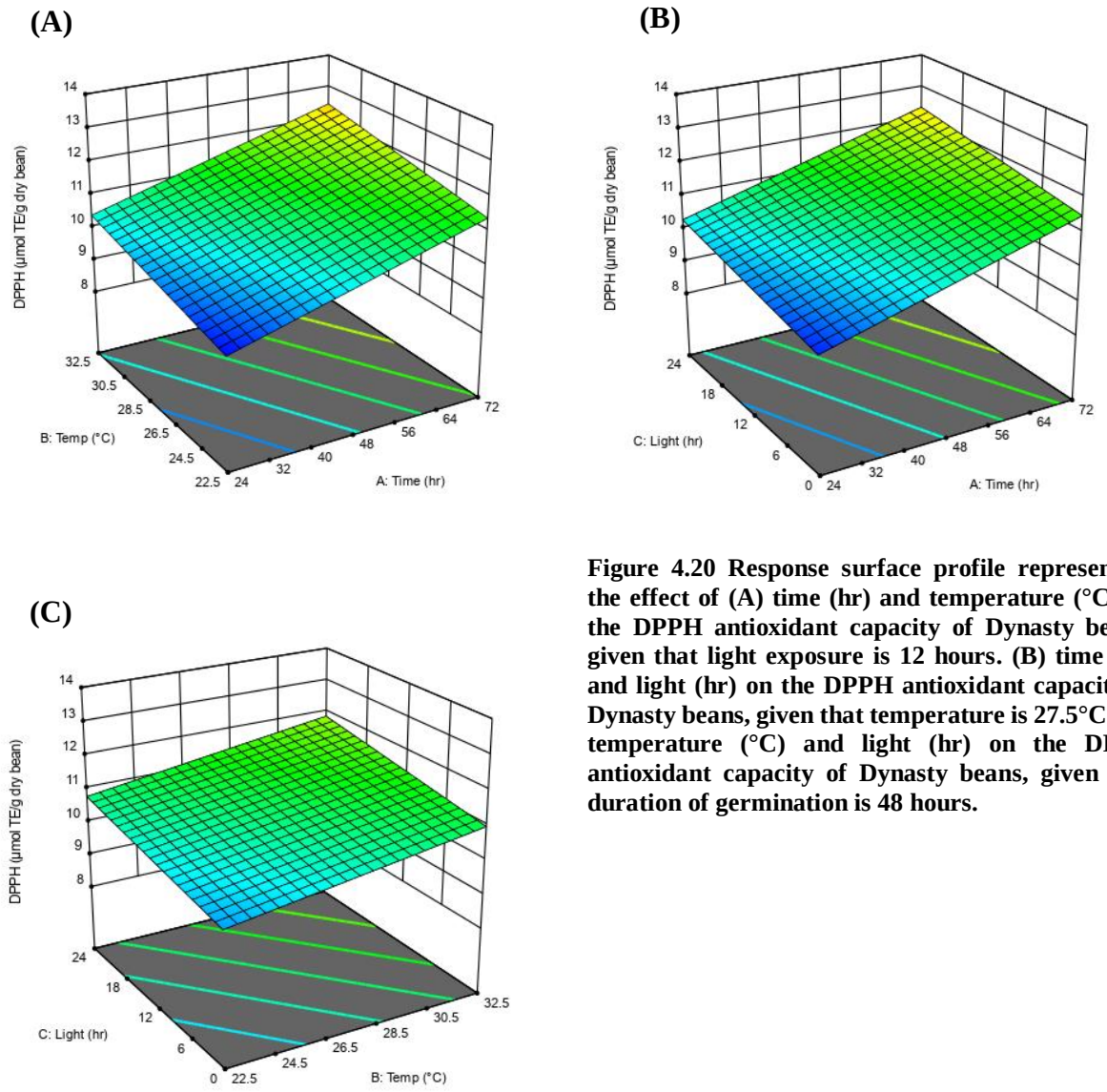


Figure 4.20 Response surface profile representing the effect of (A) time (hr) and temperature (°C) on the DPPH antioxidant capacity of Dynasty beans, given that light exposure is 12 hours. (B) time (hr) and light (hr) on the DPPH antioxidant capacity of Dynasty beans, given that temperature is 27.5°C. (C) temperature (°C) and light (hr) on the DPPH antioxidant capacity of Dynasty beans, given that duration of germination is 48 hours.

The difference between ABTS and DPPH values can be attributed to several factors. The first is due to the stability of the two radicals. DPPH tends to produce lower antioxidant capacity values, since it is more stable (and hence, less reactive) than the ABTS radical (Mareček et al. 2017). The phenolic acid profile of the food also has an effect on the obtained antioxidant capacity values. According to Kaneda et al. (1995), while DPPH radical reacts well against polyphenols such as catechins and proanthocyanidins, it doesn't react as well against phenolic acids such as caffeic and

ferulic acid. This is relevant considering the phenolic acid profile of *Phaseolus vulgaris* contains higher amounts of catechin over caffeic and ferulic acid (Ramírez-Jiménez et al. 2014). The extract solvent is shown to have a significant effect on the antioxidant capacity values obtained from ABTS and DPPH assays (Pérez-Jiménez and Saura-Calixto 2006). Fat-soluble phenolics like flavonoids may not be as reactive in a polar solvent like water, compared to a methanolic solvent. Finally, the reaction kinetics between the two radicals has an effect on their antioxidant capacity values (Campos and Lissi 1997).

4.2.6.1 Comparison of TEPC to Antioxidant Capacity

TEPC and antioxidant capacity were plotted to observe the correlation between the two values.

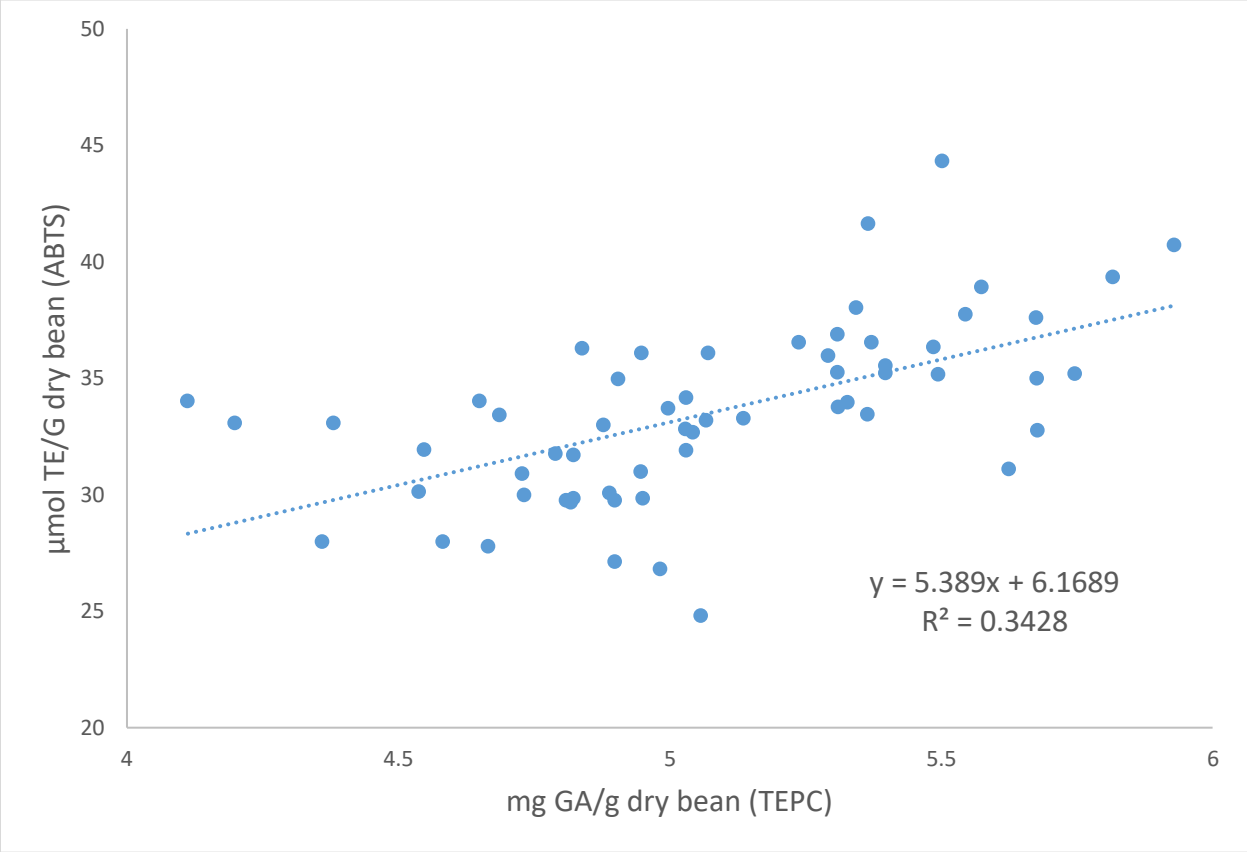


Figure 4.21 Correlation between TEPC and ABTS values

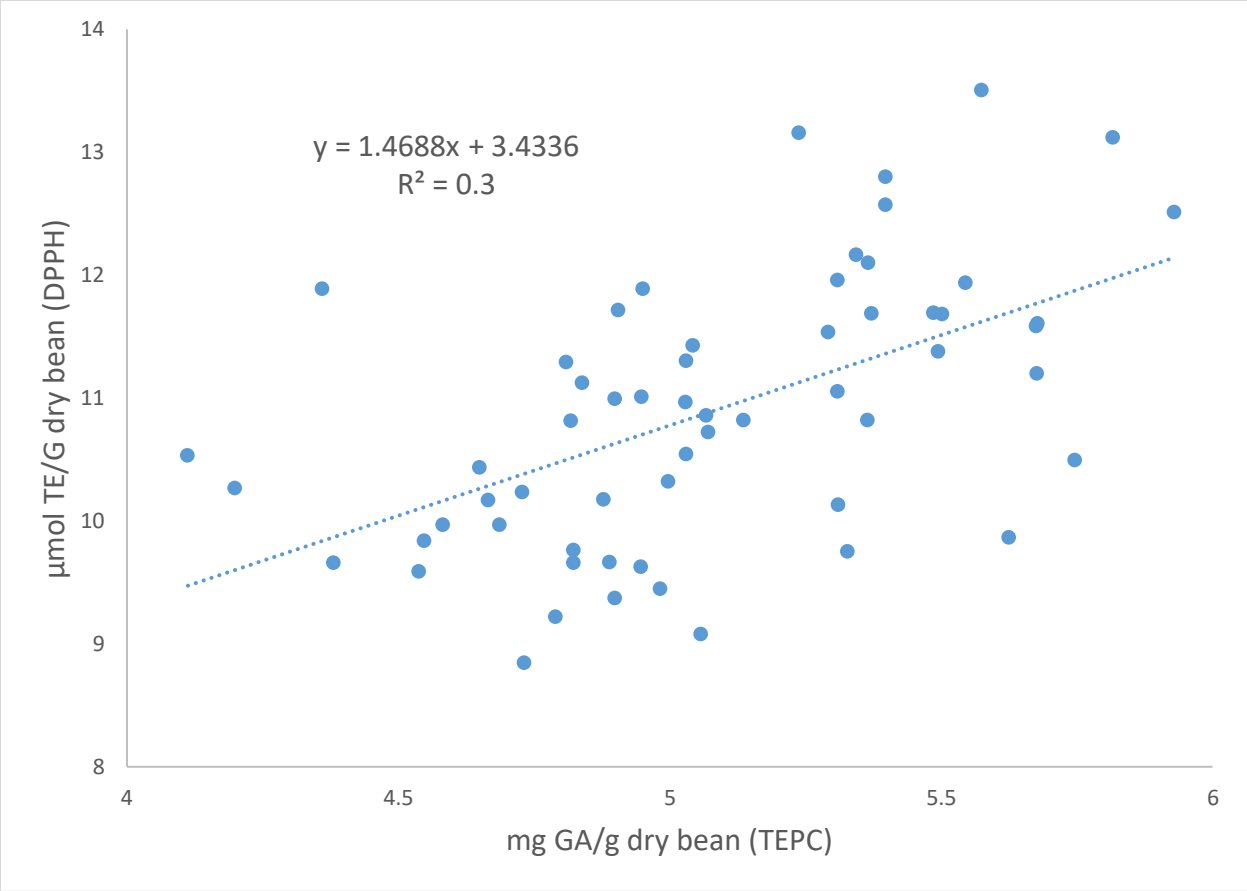


Figure 4.22 Correlation between TEPC and DPPH values

It was found that there was not a strong correlation between TEPC and ABTS, and between TEPC and DPPH values (Figure 4.21, Figure 4.22). This suggests that the extraction method for TEPC and/or antioxidant capacity was incomplete, resulting in artifacts in the final value.

4.2.7 Optimization of Germination

From the models developed in the previous sections, the germination process can be optimized based on several, but not all of the responses. For the soluble sugars (sucrose, galactose, glucose, fructose), these models are somewhat adequate, since for all models the difference between their R^2 and adjusted R^2 is less than 0.2. However, their models were not considered for optimization since their R^2 values were quite low (0.52-0.65). For protein, protein digestibility, resistant starch content, and TPC, the models were found to not be statistically significant enough to merit

inclusion in the optimization process. The R^2 values of these models were 0.47, 0.29, 0.48 and 0.30 respectively, and although these models had a good agreement of their R^2 value with their adjusted R^2 value (except for resistant starch content), their overall low R^2 value makes these models unfit for inclusion in the optimization process.

Thus, for the optimization of the germination process, three responses were selected. The first response is total alpha-galactosides, since their reduction must be facilitated in order to develop a functional ingredient for patients with gut diseases. ABTS and DPPH antioxidant values were also used in optimization, since their model was significant and had a good fit, and because their R^2 values were in agreement with their adjusted R^2 values.

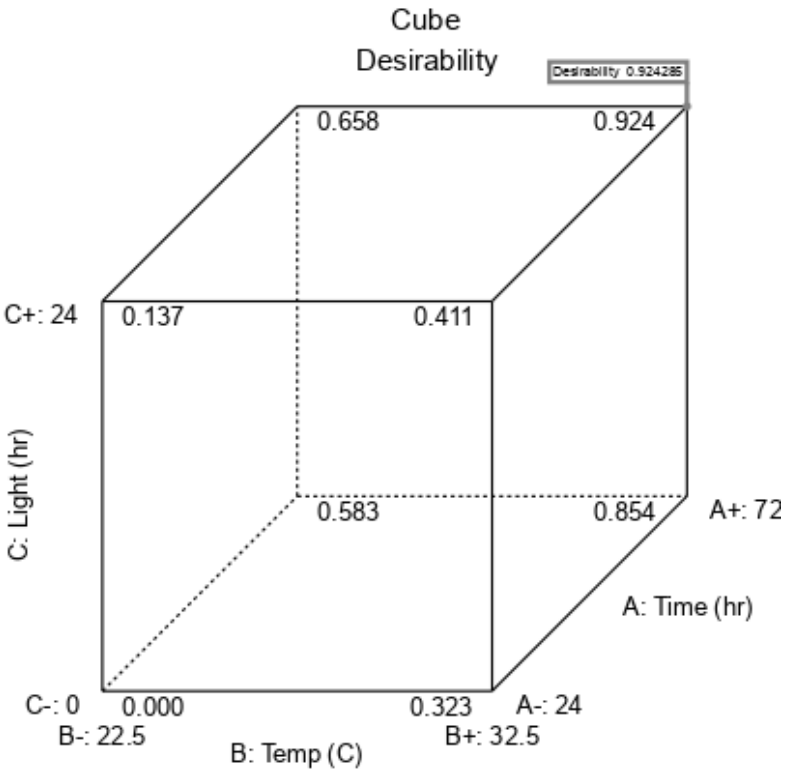


Figure 4.23 Desirability cube featuring the desirability of each combination of level extremes

The optimization indicates that germination of beans at 72 hours, 32.5℃ ambient temperature, under full light (24 hours), would result in beans that have significantly reduced total alpha-

galactosides, as well as significantly increased antioxidant capacity. This combination of factors has the highest desirability score (0.924), indicating that it is the most feasible combination of levels for the given factors (Figure 4.23). This is logical given that for all three responses, their maximum or minimum was recorded at the higher extremes of time and temperature, and in the case of DPPH, at the high extreme of light. It was hypothesized that the optimal process conditions would be at the midpoint of each variable (48 hours, 27.5℃, 12 hours of light), however this was shown to be false, as increasing conditions only led to further reductions in alpha-galactosides and increase in nutritional values.

While the maximum reduction of total alpha-galactosides was actually predicted to be at the 60hr mark, instead of the extreme (72 hours), overall the optimized process favors a germination process with a duration of 72 hours to achieve the most optimized ABTS and DPPH response. In an industrial scale germination process, the industrialist may want to choose the 72 hour process over the 60 hour process, since the extra 12 hours of germination (and thus the extra 12 hours of resources consumed) would result in a higher antioxidant capacity of the finished bean, which would produce a bean powder that is more attractive to the consumer. Another factor to consider is that flatus production increases linearly with alpha-galactoside content, and thus it is in the industrialist's best interest to completely remove alpha-galactosides from the bean, so that flatus is not produced at all when the bean powder is consumed (Reddy, Salunkhe, and Sharma 1980).

5 Conclusion and Future Work

5.1 Conclusion

Germination was found to reduce total alpha-galactosides in beans, as well as improve their nutritional value. The duration of germination and temperature of germination were found to be

the overall significant factors affecting the nutritional parameters and change in sugar and alpha-galactoside content of the germinated beans. Light was found to only have a significant effect on fructose content. Optimization of the germination process led to a germination duration of 72 hours, a temperature of 32.5℃, and 24 hrs of light exposure to reduce the maximum amount of alpha-galactosides, while maximizing the antioxidant capacity. The novelty of this research lies from using light as a variable in the germination process, since most studies only focused on time and temperature.

5.2 Future Work

Further research is required to explore the potential of GBF as a functional ingredient in food products. Rheological properties of the GBF, such as pasting profiles, temperature of gelatinization, and staling; physical parameters such as particle size, water and oil binding capacity; and thermal properties must be studied to evaluate the functionality of the GBF. The investigation on digestion behavior using both *in-vitro* and *in-vivo* techniques should provide valuable information about the enhanced functionality of GBF, and their potential for scale-up in food manufacturing. Finally, the sensory properties of GBF incorporated food products such as breads, pasta, or muffins must be investigated to assess the likeability and acceptability of these products.

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