Formation of Maillard reaction products and chlorogenic acid-quinone induced green pigments in gluten-free pretzels made using sunflower flour

A Thesis by

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DEDICATION

This research is dedicated to my parents – Madhu and Rakesh, who instilled the importance of education, hard work and diligence in me from a very young age. You believed in me and have made so many sacrifices for my sake – I owe this to you! I would also like to dedicate this research to the friends and professors who talked me out of quitting on numerous occasions, supported me and believed in me to complete my thesis despite my hectic life schedule. This is for you, for making sure I kept on moving forward and getting stronger, instead of giving up.

ABSTRACT

Formation of Maillard reaction products and chlorogenic acid-quinone induced green pigments
in gluten-free pretzels made using sunflower flour

by Rashi Bhatnagar

The two color-developing reactions that occur when soft pretzels are baked using sunflower flour (SFF) in aqueous alkali environment are chlorogenic acid (CGA)-quinone induced greening and Maillard browning. This study aimed to determine how CGA content in two different SFFs containing 45% and 55% protein (Heliaflor[®] 45 and 55, respectively) affected the formation of green trihydroxybenzacridine (TBA) and Maillard reaction products (MRPs) in soft baked pretzels. Pretzels were baked with five doughs prepared using a pre-blended gluten-free flour, consisting of sweet white rice flour, whole grain brown rice flour, potato starch, whole grain sweet white sorghum flour, tapioca flour, and xanthan gum: control (no SFF) and doughs replacing 3.65% and 7.30% GF flour with Heliaflor[®] (HF) 45 and 55. Pretzels were used for Hunter L, a, b color, and proximate analysis. Flour mixtures containing HF 45, HF 55, gluten free flour (GF) + HF 45, GF + HF 55 with 5% glucose, sucrose or sorbitol were also prepared. The supernatants from the centrifuged flour mixtures were used to quantify Schiff bases, CGA, melanoidins, and trihydroxybenzacridine (TBA) derivatives. The precipitates were dehydrated in a vacuum oven and FTIR analysis was used to identify changes that occurred in the secondary structures of proteins. Pretzels prepared with SFF contained CGA, whereas mixtures prepared with GF flour, without SFF, did not develop CGA and instead revealed the presence of non-CGA hydroxycinnamic acids (HCAs) such as caffeic, ferulic, and *p*-coumaric acid. The mixture containing HF 55 had the highest Schiff base, CGA, melanoidin, and green TBA content. There was no significant difference in CGA and MRPs between mixtures prepared using SFF

containing 45% vs 55% protein or between the different sweeteners used. Greening in pretzels stored at ambient temperature increased 18 hours post-baking. The quantity of SFF used can be decreased to obtain the desirable shade of green color in the pretzels. The formation of MRPs such as excessive browning and Schiff bases can be decreased by decreasing the concentration of SFF to develop a gluten-free baked pretzel containing SFF for bakeries and restaurants.

Keywords: Chlorogenic acid, FTIR, Maillard reaction, soft pretzels, sunflower flour, trihydroxybenzacridine

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LIST OF ABBREVIATIONS

3-DG – 3-deoxyglucosone – (PubChem CID: 114839)

3-APA – 3-aminopropionamide – (PubChem CID: 192802)

ACR – Acrylamide – (PubChem CID: 6579)

ATR-FTIR – Attenuated Total Reflectance – Fourier Transformed Infrared Spectroscopy

CGA – Chlorogenic acid (PubChem CID: 1794427)

FTIR – Fourier Transform Infrared Spectroscopy

GF – Gluten-free

HF 45 – Heliaflor[®] 45 (45% sunflower flour)

HF 55 – Heliaflor[®] 55 (55% sunflower flour)

HMF – 5-Hydroxymethylfurfural – (PubChem CID: 237332)

HPLC – High Performance Liquid Chromatography

MRP(s) – Maillard reaction product(s)

SFF – Sunflower flour

TBA - Trihydroxy benzacridine

LOGIC MODEL

Program: Agriculture and Food Research Initiative Program Novel Foods and Innovative Manufacturing Technologies priority code area A1364 (USDA, 2019a).

Situation: Sunflower protein concentrate or isolates contain 0.94-1.7% (d.b.) chlorogenic acid (CGA), a reactant in the Maillard, and greening reactions in baked goods (Wildermuth et al., 2016).

NEED: To understand how different quantities of protein and CGA in sunflower flour affects the formation of Maillard reaction products (Schiff bases and browning), and CGA-quinone induced greening in alkaline soft pretzels.

1. INTRODUCTION

The two color reactions that occur when soft pretzels are baked using sunflower flour in an aqueous, alkali environment are CGA-quinone induced greening and Maillard browning (S. H. Liang & Were, 2018a). An ester of caffeic and quinic acids, CGA is a phenol ubiquitous in plants such as coffee beans, pears, apples, and sunflower seeds (Wang et al., 2019; Wildermuth et al., 2016). At alkaline pH and in the presence of amino acids, CGA oxidizes and forms green color adducts. Oxidized CGA forms CGA-quinones which are unstable and easily react with amino acids, and upon nucleophilic cyclization with amino or thiol groups of proteins and loss of water, forms green trihydroxy benzacridine (TBA) derivatives (Bongartz et al., 2016; Weisz, Kammerer, & Carle, 2009; Wildermuth et al., 2016; Yabuta, Koizumi, Namiki, Hida, & Namiki, 2001).

Means of mitigating greening in dough and cookies prepared using sunflower butter have been explored (Atonfack, Ataman, & Were, 2019; S. Liang, Tran, & Were, 2018; Rogers, Hahn, Pham, & Were, 2018). These mitigation strategies include the use of acidic ingredients or egg replacers, decreasing moisture content, and baking temperatures to lower greening (Atonfack et al., 2019; S. H. Liang & Were, 2018b; Rogers et al., 2018). When maple syrup, a sweetener more basic and with higher moisture content than honey (2% w/v; pH 6.28 and 3.93, respectively) was used in cookies baked with sunflower butter, post-baking green color formation was higher, compared to cookies baked using honey (Atonfack et al., 2019; S. H. Liang & Were, 2018a). Rogers et al. (2018) studied the effects of using egg and two plant-based egg replacers (chia and bananas) with varying protein and moisture contents, and baking cookies prepared using sunflower butter at two temperatures (162.8°C and 190.6°C). Cookies baked with egg had the highest moisture content due to the humectancy of egg phospholipids (3.0% lecithin in egg

yolks), which helped contain the moisture within the cookie while baking and prevented moisture loss as a result of cracking as occurred with cookies baked with bananas. Additionally, cookies baked at the lower temperature (162.8°C) had the highest internal greening as a result of the higher moisture content than cookies baked at 190.6°C, where there was more moisture loss as a result of baking.

The reducing sugars and protein in the cookies also form aroma and brown Maillard reaction products (MRPs). Soft pretzels are baked using sucrose, a non-reducing sugar, but sucrose can degrade to fructose and glucose under alkaline conditions at 185- 190 °C within two hours (Van der Fels-Klerx et al., 2014), contributing to Maillard browning. Chlorogenic acid also contributes to Maillard browning. When mixtures comprising fructose alone, fructose and aspartic acid (pH of 5.5 or 7), and glutamate and glucose were heated in the presence of CGA, the formation of hydroxymethylfurfural (HMF), one of the many Maillard reaction products, increased with increase in CGA concentration (Cai et al.; Jiang et al., 2013; Z. H. Zhang et al., 2016). Furthermore, Cai et. al., (2014) found that adding free CGA to an equimolar asparagine/glucose mixture (pH 6.8) increased the formation of HMF and acrylamide (ACR), whereas CGA-quinone derivative decreased ACR formation. An increase in CGA led to HMF formation, which further promoted the development of ACR in the mixtures.

Before baking, soft pretzels are dipped in a baking soda or lye bath, resulting in an alkaline surface, providing the ideal environment for both, Maillard browning and CGA-quinone induced surface greening. An understanding of whether CGA from sunflower flour is contributing more or less to the formation of Maillard browning versus chlorogenic-induced green color in an alkaline baked good is lacking.

The relationship between CGA naturally occurring in sunflower flours (SFF) and the

formation of brown melanoidin compounds in an alkaline environment, when partially replacing gluten-free flour (GF) with SFF, requires investigation. Understanding this relationship would help determine how a natural green color can be developed, while the concurrent development of browning occurs (Nguyen, Van der Fels-Klerx, Peters, & Van Boekel, 2016).

The overall research goal was to develop a natural green non-allergenic gluten-free soft pretzel using sunflower flour. The specific objectives were to determine (I) if CGA and protein from sunflower flour in combination with a gluten-free flour blend promotes the formation of green TBA derivatives and (II) the extent to which the different concentrations of sunflower flour, high temperature and pH and time of storage post-baking contribute to the formation of green TBA derivatives and the formation of MRPs. In a moist, alkaline environment, CGA from sunflower flour contributes to the formation of green TBA derivatives and MRPs, some of which are undesirable, such as HMF and acrylamide (ACR) (Cai et al., 2014; Z. H. Zhang et al., 2016). It was hypothesized that if Heliaflor[®] 55, sunflower flour containing a higher protein content (55%) versus Heliaflor[®] 45 (45% protein) is used to replace the GF flour, then less free CGA is present to react in the greening reaction. Using a lower concentration of the sunflower flour in pretzels (3.65% versus 7.30%) can decrease the formation of MRPs as available CGA substrate is expected to be lower when a lower concentration of 3.65% is used. However, the presence of reducing sugars, such as glucose, and components of the GF flour blend can still react to form MRPs (Q. B. Zhang, Ames, Smith, Baynes, & Metz, 2009). According to Yabuta et al. (2001), when an alkaline system containing CGA, without amino acids, was aerated, browning increased. Chlorogenic acid quinones may also contribute to browning in addition to browning from the Maillard reaction. Lastly, it was hypothesized that since soft-baked pretzels have a more alkaline surface from being dipped in a baking soda/lye bath prior to being baked, Maillard

browning and greening will be favored on the surface more than the interior of the pretzel. By understanding the relationship between CGA and its effect on greening and formation of MRPs in the GF soft pretzels with sunflower flour, the amount of sunflower flour used to prepare GF soft baked pretzels can be adjusted to decrease the amount of free CGA available as a reactant in the Maillard reaction. Hence, the potential formation of excessive browning can be decreased, and CGA-quinone derivatives (Cai et al., 2014) increased, promoting the formation of natural green pigment in pretzels sold at bakeries, for pastry chefs and restaurateurs who want to produce gluten-free baked goods using sunflower flour for special holidays, such as St. Patrick's Day, Oktoberfest, Halloween, and Christmas.

2. LITERATURE REVIEW

2.1 Advantages and current applications of sunflower products in the food industry

There is an increased interest amongst consumers towards adapting sunflower flour as shown by the success of companies such as Moxie Bread Co. (Louisville, CO) and the Planetarians food company (San Francisco, CA) who are currently incorporating sunflower meal and sunflower flour in their recipes. Consumers increased interest in plant-based alternatives to animal-based proteins is due to their association with positive health effects, such as weight control and cardiac health. According to a 2017 survey by Nielsen International, LLC, 23% of consumers demanded additional plant-based protein options (CPG, 2017). Consumers are willing to explore plant-based protein sources other than soy and pea, such as lentils, nuts, and sunflower (Nielsen, 2017). Sunflower meal (SFM), which is the byproduct of sunflower (*Helianthus annuus*) oilseeds' extraction, is a non-allergenic, non-genetically modified source of protein, higher in crude fiber (21%) than soy protein (3.1%). Although soybean meal has a higher crude protein content (52.9 %) than sunflower meal at 35.0% (Anderson & Lardy, 2012), soy protein

has its drawbacks including its potential allergenicity in 0.5% and 0.6% of the children and adults in the US populations, respectively (Gupta et al., 2018; Gupta et al., 2019). Additionally, 94% of soybeans are genetically modified (Dodson, 2019), while sunflower seeds are typically not genetically modified (Anderson & Lardy, 2012).

Sunflower meal is a by-product of sunflower oil production and is grown globally with India, China, EU, US, and Turkey being the major producers. Depending on the method used for oil extraction (mechanical or solvent), the fat content of SFM varies from 1% with solvent to 13% with mechanical extraction, which also alters the protein content of SFM, ranging from 23-40%, depending on the defatting methods used. Unlike pea and other legume-based proteins, sunflower protein has a mild, nutty flavor, and is an economical source of protein due to its comparatively lower lysine content and high fiber content. Although it is not free from anti-nutritional factors such as trypsin inhibitors when compared to soybean flour, sunflower protein has less trypsin inhibitor activity, although this may vary based on the sunflower variety (Salgado, Ortiz, Petrucci, & Mauri, 2011; Wildermuth et al., 2016)

Companies, restaurateurs, chefs, and bakers are incorporating sunflower seed and oil by-products in their foods. The Planetarians food company (San Francisco, CA, USA) upcycled defatted sunflower seeds to produce a protein enriching SFF ingredient, that is included in a variety of finished foods such as sunflower chips, breads, noodles and pastas, meat and sweet biscuits (Planetarians, 2019). Planetarians is currently working with the Barilla Center for Food and Nutrition (Parma, Italy) to develop pasta, snacks, baked good, meat, dips, purees and breakfast items using the SFF (Siegener, 2019). The chef of Ice CREAM Social (New York City, NY, USA), Anna Fitting uses Planetarians' SFF in her cakes, buns, pizza crust, and as a component in the cake in her ice cream sandwiches (Berry, 2019). Moxie Bread Co. (Louisville,

CO) uses sunflower seed flour to replace one-third of the wheat flour to prepare their vegan/vegetarian pizza crusts owing to its added fiber content (Berry, 2019). Hudson Valley Cold Pressed Oils (Pleasant Valley, NY, USA) also launched a gluten-free sunflower flour, in addition to gluten-free sunflower brownie and “brunette” mixes (Watrous, 2019). According to Hudson Valley Cold Pressed Oils, the SFF can be used as a nutty flavor enhancer and as a 1:1 replacement for almond flour in some baking applications.

2.2 Chlorogenic acid (CGA) and protein content of sunflower flour

Sunflower seeds are comprised of 20-30% protein and are rich in sulfur-containing cysteine and methionine and limiting in lysine (Karefyllakis, Salakou, Bitter, van der Goot, & Nikiforidis, 2018; Wildermuth et al., 2016). Sunflower seeds have lower protein content than soybeans of 22 versus 35% (GmbH, 2014). However, the protein and fat content of the sunflower flour can be altered depending on the method of oil processing (mechanical or solvent extraction).

The main phenolic compound in sunflower seeds is chlorogenic acid (CGA), with whole seeds containing one of the highest CGA contents at 1.1-4.5% on a dry mass basis compared to 0.5-2.4% in the dehulled seeds (Gonzalez-Perez, 2007). Despite green coffee beans having the highest CGA content (6-12%), between 30-100% of CGA is lost during roasting, depending on duration (Farah, De Paulis, Trugo, & Martin, 2005), as the carbon-carbon bonds in the molecule break, dehydrating the chlorogenic acid to lactones.

When compared to legumes, tubers, and wheat, sunflower seeds contain the least carbohydrates (Brecht, Ritenour, Haard, & Chism, 2008). **Table 1** summarizes the percent macronutrients and CGA in various seed crops and their effect on greening and MRPs.

2.3 Current limitations of using sunflower flour in the food industry

Due to its health and economic advantages, it is forecasted that the global market for sunflower meal will grow at a CAGR of 6.05% between 2019-2024 (Mordor, 2019), which in turn will increase sunflower flour and protein availability. Despite its higher concentrations of sulfur-containing cysteine and methionine, SFF is limiting in lysine. However, this can be complemented by combining SFF with lentil and pulse proteins. If SFM is used as a food ingredient without further processing to extract the proteins, the high fiber content in SFM can interfere with emulsion formation by sterically hindering the interfacial activity of proteins, rendering them unable to reduce the interfacial tension when forming an emulsion (Karefyllakis, Octaviana, van der Goot, & Nikiforidis, 2019). However, using SFF with a higher protein and lower fiber content can aid in the formation of emulsions as it can function as a Pickering stabilizer. The last limitation with using SFF in the food industry is because its use can lead to the formation of green pigment as a result of its chlorogenic acid (CGA) oxidizing in an alkaline environment.

The total global production of sunflower seed oil increased from 15.39 million metric tons in 2015/16 to 19.81 million metric tons in November 2019, resulting in more SFM. The total production of SFM, a by-product of sunflower oil produced from oil-type sunflower seeds, increased from 16.51 million metric tons in 2015/16 to 21.26 million metric tons by November 2019 (USDA, 2019b). A quarter of non-oil-type sunflower seeds are currently used as birdfeed, and 10-20% of the non-oil-type sunflower seeds are sold to the baked goods and snacks industry to be roasted and used in nutrition bars (Ash, 2017). Instead of being fed to livestock, SFM rich in fiber, CGA, minerals, and protein can be used to produce sunflower flour, which contains fewer anti-nutritive factors, such as trypsin inhibitors (Sosulski, 1979; Wildermuth et al., 2016).

Table 1. Proximate analysis and chlorogenic acid content (%) of various seed crops and flours

Seed Crops and Flours	Proximates (%)			Chlorogenic Acid content (%)	Effect of proximates and CGA on Greening and MRP formation	Reference
	Protein	Carbohydrate	Lipid			
Peanut¹	28	15	50	0.0033– 0.0044	Contains comparatively low CGA: no significant effect on formation of CGA-quinone greening. Higher carbohydrate content than sunflower protein concentrate, isolate, and flours may contribute to more MRP formation.	(Cho et al., 2010)
Soybean¹	35	25	21	0.0078 – 0.0221	Lower concentration of free CGA than sunflower concentrates. Higher carbohydrate content than peanut and higher protein content than peanut, chickpea, pea, and wheat can increase formation of MRP's than aforementioned seed crops.	(Cevallos-Casals & Cisneros-Zevallos, 2010; Giusti, Caprioli, Ricciutelli, Vittori, & Sagratini, 2017; Wildermuth et al., 2016; Zhu et al., 2018)
Sunflower¹ protein concentrate and isolate	35	5	45	0.94 - 1.7	Highest concentration of free CGA (d.b.) compared to other oilseeds contributes the most to CGA-quinone induced greening and formation of MRPs.	(Wildermuth et al., 2016)
Heliaflor[®] 45^{2*}	45	7	10	0.94 – 1.7	Free CGA content is similar to that of Heliaflor [®] 55. No significant difference in greening and browning was found when used at same concentration as Heliaflor [®] 55.	(Austrade, 2015; Wildermuth et al., 2016; Current study)
Heliaflor[®] 55^{2*}	55	9	2	0.94 – 1.7	Should contain less free CGA than Heliaflor [®] 45, owing to its higher protein content. No significant difference in greening and browning	(Austrade, 2015; Wildermuth et al., 2016; Current study)

was found when used at same concentration as Heliaflor® 45.

Chickpea¹	21	71	5.0	0.0111	MRP formation due to carbohydrate and protein (lysine) content.	(Giusti et al., 2017; Mohammed, Ahmed, & Senge, 2014; Moussou et al., 2017; Nikolic, Todorovic, & Lazic, 2011)
Pea¹	25	70	2.0	0.000057-0.00013	May contribute to HMF production when thermally processed due to 70% carbohydrate content. Higher contribution to formation of MRPs than chickpeas. Chlorophyll is responsible for the green color, not CGA-quinone induced greening. Presence of other phenolic acids (gallic, ferulic, <i>p</i> -coumaric) may contribute to greening.	(Moussou et al., 2017; Stanisavljevic et al., 2016)
Wheat gluten¹	12	80	2.0	0	81% carbohydrate and 12% protein with thermal processing (baking) contributing to high MRP formation. Wheat does not contain CGA, so does not form green color.	(Shchekoldina & Aider, 2014)

This table was adapted from ¹(Brecht et al., 2008) and ²(Austrade, 2015)

*Heliaflor® is the processed organic sunflower Flour, produced by peeling sunflower kernels and partially defatting them using a mechanical press. It was purchased from Austrade (Austrade, 2015)

2.4 Sunflower protein functionality in gluten-free pretzels

The gluten-free industry was \$1.7 billion in 2011, grew to \$3.5 billion in 2016, and it is expected to reach up to \$4.7 billion in 2020 (McCarthy, 2017). Approximately 1% of the Western population is affected by celiac disease (Hughey et al., 2017; Rewers, 2005). Of the 3.1 million people in the United States that avoid gluten, 72% are considered “PWAGs” (people without celiac disease avoiding gluten), who have adopted a gluten-free diet due to gluten sensitivity or as precautionary measures against irritable bowel syndrome or gastrointestinal discomfort (McCarthy, 2017). The development of gluten-free baked goods accommodates those who follow a gluten-free and low FODMAP diet (Lis, Kings, & Larson-Meyer, 2019).

In terms of solubility and its functionality as an emulsifier, sunflower protein is a potential alternative to allergenic proteins such as soy protein, as sunflower protein exhibits interfacial and emulsifying activity, thickening, water, and fat binding as other plant-based proteins (Karefyllakis et al., 2018). In addition to the functionalities of eggs and starches and gums found in the GF blend used to make baked goods, the water-binding and thickening functionalities of protein in sunflower flour can aid in GF soft pretzel dough formation. Pre-blended starches such as rice, potato, and tapioca are available for home-bakers to make a variety of GF baked products, including pretzels. Crawford, Kahlon, Chiu, Wang, & Friedman (2019) found that the protein and starches in various GF blends can be functional when preparing gluten-free flatbreads. Adding sunflower flour to the GF blend may increase the protein content of the baked pretzels while providing emulsification that may otherwise not be sufficient from starches in the GF blend. Lecithin found in egg yolks also provides additional emulsification, to compensate for the decreased protein in GF flour (Bongartz et al., 2016). The owner of Moxie Bread Co. (Louisville, CO), Andy Clark, uses sunflower flour in his pizza crusts for its fiber and

protein contents. The fiber from the sunflower seed flour provides additional water binding and produces a dough, with boosted nutrition profile and an appealing green color, which “reinforces the mindful choice” (Berry, 2019).

2.5 Role of alkaline pH and water in gluten-free soft baked pretzels

Pretzels are alkaline baked foods, which provide an optimal environment for CGA-quinone induced greening. Greening may lower the availability of free CGA to react with α -dicarbonyls, hence decreasing the formation of MRPs. However, if higher quantities of sunflower flour are used (7.30% versus 3.65%), the formation of hydroxymethylfurfural (HMF) and acrylamide (ACR), which can form at temperatures above 120 °C in the presence of 1- or 3-DG and Asparagine (Asn), respectively, can increase with increased CGA content (**Figure 1**).

Water is critical to the formation of gluten-free soft pretzels, as it plasticizes and hydrates the gluten-free blend flours. A higher water content is required when developing gluten-free versus gluten-containing baked goods, as the fine starch particles have a larger contact surface area requiring more water for hydration and the eventual swelling of the starches comprising the GF flour blend (de la Hera, Rosell, & Gomez, 2014). If the starches are not hydrated sufficiently, they will not contribute to the formation of structure in gluten-free pretzels. Water is also added for the activation of yeast, resulting in the leavening of the dough and pretzels.

Figure 1. Colored chemical reactions and associated products formed in soft-baked pretzels made using sunflower flour

2.5.1 Role of alkaline pH in the formation of Maillard reaction products

Depending on the pH and reactants involved, Maillard reaction takes different routes, which determines the final products developed. Under heat and alkaline environments, sucrose fragments to glucose and fructose, promoting the formation of MRPs including ACR (Nguyen et al., 2016), at temperatures of 120°C and higher, i.e., baking and frying (De Vleeschouwer, Van der Plancken, Van Loey, & Hendrickx, 2006). In an alkaline environment, the sugars are in their open-chain reducing form, which promotes reactivity with amino acids resulting in browning and flavors associated with the Maillard reaction (Mikami & Murata, 2015). Additionally, under alkaline conditions, highly reactive reductones, and dicarbonyls form and promote the formation of advanced MRPs (Ajandouz, Tchiakpe, Ore, Benajiba, & Puigserver, 2001).

Zhang et al. (2016) found that CGA promoted the formation of HMF when fructose was heated in acidic or neutral pH environments by enhancing production of the HMF precursor, 3-deoxyglucosone (3-DG), and converting it to HMF in the Maillard reaction. Jiang et al. (2013) found that CGA promoted HMF formation when pH was adjusted to neutral in a glucose/glutamate mixture, but HMF was depleted in other glucose-amino acid mixtures, namely lysine and cysteine. Jiang et al. (2013) suggested that HMF depletion occurred from the reaction between the ϵ -amine group of lysine and sulfhydryl group of cysteine leading to the depletion of the MRP.

Pretzels are dipped in an alkaline solution to develop a smooth, dark brown surface. The shiny surface develops as a result of the gelatinization of starch granules in an alkali medium, the dissociation of amylose-lipid complexes, and a decrease in reducing sugars (Rombouts, Lagrain, Brijs, & Delcour, 2012), which upon baking at high temperatures results in the characteristic hardened shiny surface. The proteins in the pretzel dough hydrolyze and the amino compounds,

in the presence of monosaccharides and other carbonyl compounds, produce brown Maillard melanoidins, decreasing reducing sugars on the surface of the dough post-alkaline dip (Ni, Owusu-Apenten, Zhu, & Seetharaman, 2006).

2.5.2 Role of alkaline pH on green trihydroxy benzacridine formation

Under alkaline environments, CGA oxidizes to form highly reactive *o*-quinones, which bind with reactive sites on free NH₂ groups in sunflower flour (S. H. Liang & Were, 2018b) to produce green trihydroxy benzacridine (TBA) derivatives (Bongartz et al., 2016; Karefyllakis et al., 2018; Wildermuth et al., 2016).

At alkaline pH, depending on the amino acids present in the mixture, formation of oxidized CGA-induced greening, as demonstrated in **Figure 1**, results from the exposure of more binding sites on the proteins, increasing the formation of covalent bonds between the proteins in sunflower and CGA.

2.5.3 Role of moisture on promoting Maillard browning and chlorogenic-induced greening

Increased water activity can enhance sunflower protein-CGA binding due to increased mobility of the reactants, increasing the molecular interactions between CGA and protein (S. H. Liang & Were, 2018b; Rogers et al., 2018; Wildermuth et al., 2016). Wildermuth et al. (2016) observed increased surface greening in cookies made using sunflower butter and maple syrup that were stored in Ziploc[®] bags versus those left in the open, due to condensation in the bags, increasing the moisture content and reactivity of precursors inducing greening.

2.6 Color and texture reactions in other alkaline foods

Although thermal processing is not always necessary for the Maillard reaction and can take place in an alkaline environment, baking accelerates the formation of MRPs and browning.

Alkaline baked foods include lye bread rolls, which have a pH of 8, similar to soft-baked pretzels as they are made using the same ingredients and dipped in a caustic bath (West & Cornwell, 2002). Lye bread rolls and soft-baked pretzels, however, differ in shape. Both lye bread rolls and soft-baked pretzels have uneven surfaces, where the folds at the pretzel knots and the uneven surface of the lye bread rolls can affect development of greening or MRPs. Other alkaline foods include *masa harina*, made from dried corn treated with lime in a nixtamalization process. The pH of *masa harina* ranges between 7 and 8. Masa is thus usually not substituted for corn starch as a thickener in baking applications due to its alkaline pH (Rosentrater, Flores, Richard, & Bern, 1999) and is generally used to make *tamales* and corn tortillas. *Masa harina* is white in color, unlike most cornmeal consumed in the United States, which is yellow and has a grainy texture. However, cornmeal can be ground and used as a substitute to *masa harina* as needed.

Mooskuchen, also known as “moss cake” is a Central European cake made with a topping of ground coffee beans and egg whites, which provide a neutral pH (Carle & Schweiggert Ralf, 2016). The CGA from the coffee beans reacts with the egg white proteins, resulting in a vibrant green color (Carle & Schweiggert Ralf, 2016). The green *Mooskuchen* topping remains bright green owing to the neutral or basic pH of 7.0 - 9.0 provided by the egg whites. At neutral pH, minimal exposure to light, and continuous storage at ambient temperature, the green chromophore is stable (Carle & Schweiggert Ralf, 2016). Lastly, *Lutefisk*, whose pH ranges between 11 to 12, is a traditional Nordic dish made from air-dried whitefish and lye to produce a gelatinous texture (Lunestad, Grevskott, Roiha, & Svanevik, 2018).

Consumers’ demands for healthier snack options containing higher protein has increased since 2014. Consumers want to see more “clean labeled” fortified foods, which offer immunity,

boost energy, and have enhanced protein (Fasolin et al., 2019). One small (62 g) unsalted soft-baked pretzel contains 15% total carbohydrates and 3% total fats but are lacking in protein (5.1 g/ 62 g serving) (Nutritionix, 2016). Additionally, other than the 1% of Western population that has Celiac disease, the PWAGs contribute to the “halo effect” of associating gluten-free with healthy (Popp & Maki, 2019; Rewers, 2005). This population has self-diagnosed and self-reported wheat sensitivity leading to an increase in sales of gluten-free products. More than patients with Celiac disease, the sales of gluten-free products has increased owing to the self-reported gluten-sensitive population (Niland & Cash, 2018). Hence, a gluten-free soft pretzel with increased protein content was developed and used for further analysis.

2.7 Processing sunflower flour from defatted sunflower meal

Sunflower meal (SFM) is a by-product of oil extraction of dehulled oil-type sunflower seeds, containing a high protein and fiber content. Some food companies, such as Planetarians, have recently began using defatted sunflower meal as an ingredient, despite its previous used solely as feed for ruminants, due to its unappealing dark color, which develops as a result of the high CGA content (Ivanova, Chalova, Koleva, Pishtiyski, & Perifanova-Nemska, 2012; Wildermuth et al., 2016).

Austrade, Inc has developed a method for obtaining beige and white Heliaflor[®] 45 and 55 in which the protein and phenolics are both preserved in the final flour (Austrade, 2015; **Figure 2**). The production of HF 45 and 55 both begin using shelled sunflower seeds, which are defatted using mechanical cold-pressing. Austrade, Inc. uses the patent pending Kryonert technology where heat and solvents are not used to obtain the sunflower flours. This preserves the phenolics and ensures that the protein is not denatured. The processing of HF 55 involves an additional CO₂ extraction step, producing a lighter-colored flour than HF 45, with an increased protein and

decreased fat content. The use of CO₂ for extraction is used as an alternative to solvent extraction, owing to its versatility as a solvent when temperature and pressure are altered, to remove fat from the sunflower seed cake (Arab et al., 2019).

Figure 2 Defatted sunflower meal processing, using cold-pressing and CO₂ extraction to obtain Heliaflor® 45 and 55 sunflower flours by Austrade, Inc (Austrade, 2015).

The two flours differ in color - Heliaflor® 45 is naturally darker than Heliaflor® 55 - as shown in **Figure 3**. Heliaflor® 55 is processed from Heliaflor® 45, which undergoes the additional CO₂ extraction step, done to increase the protein content and reduce the fat content of the flour (Austrade, 2015). The difference in the fat and protein values are later confirmed and shown in **Table 3**.

Figure 3. Heliakor® 45 (45% SFF) (left) has a noticeably darker color than Heliakor® 55 (55% SFF) (right) owing to the higher carbohydrate content in Heliakor® 45

Since HF 55 contains approximately 10.5% lower fat than HF 45, it has increased fat-binding when used in food applications (Austrade, 2015). The preservation of phenolics in the finished flour may, however, contribute to the decreased solubility, digestibility, and PDCAAS score of the sunflower flour as a result of the phenol-protein complexes that may form (Karefyllakis et al., 2018).

2.8 Mechanism of chlorogenic acid induced green pigment formation in sunflower flour

Phenolics can oxidize to quinones at elevated temperatures in the presence of light, enzymatically by peroxidases, polyphenol oxidases (PPO) such as catechol oxidase or laccases, or non-enzymatically in an alkaline environment (Karefyllakis et al., 2018; Prigent et al., 2008; Schieber, 2018). The hydroxyl group on the phenol is deprotonated owing to increased pH, leading to the formation of quinones. The quinones are electron-deficient highly reactive intermediates that can covalently react with protein nucleophiles, including the ϵ -amino group of lysine, sulfhydryl group of cysteine, and indole nitrogen ring of tryptophan. The oxidized phenol and protein nucleophiles form C-N and C-S bonds in an irreversible addition reaction to form various adducts (Karefyllakis et al., 2018). The CGA in sunflower flour covalently complexes with proteins, leading to indigestible proteins, decreased solubility and formation of green

chromophores. Bongartz et al., (2016) found that the alkaline treatment of sunflower meal solutions containing amino acids with reactive open sites, resulted in the formation of six green trihydroxy benzacridine (TBA) derivatives (**Figure 4**) after CGA-quinones and free NH₂ groups of amino acids form covalent bonds.

Figure 4. Transformation of chlorogenic acid to **Trihydroxy benzacridine** derivatives under non-enzymatic conditions.

The green TBA pigment does not form when amino acids react with monomers of CGA. They form with the oxidized CGA/ CGA-quinones. Not all amino acids react with CGA quinones to form greening. The ε-amine side group of lysine is highly reactive as it readily cross-links with CGA-quinone to form green TBA derivatives (Bongartz et al., 2016). Cysteine, which is highly nucleophilic amino acid, reacts with the CGA-quinone, and instead of forming green TBA, it results in colorless adducts (Y. Liang & Were, 2019). Chlorogenic acid-quinone, an ortho-diphenol with a carbonyl side chain, and a primary NH₂ group on the amino acid are required for the green pigment formation (Bongartz et al., 2016; Yabuta et al., 2001).

Chlorogenic acid in cookies made with sunflower butter decreases post-baking due to the formation of CGA-amino acid adducts/TBA derivatives (Rogers et al., 2018). However, it is

unclear as to how much CGA contributes to the formation of MRPs and browning versus how much CGA contributes to the formation of green color.

2.9 Non-enzymatic Maillard browning in baked pretzels

The concentrations of various carbonyls (oxidized CGA-quinone or reducing sugars), amino acids, and leavening agents affect Maillard browning. In addition to reactant concentration, thermal processing and pH also contribute to different Maillard browning products (Nguyen et al., 2016).

In addition to producing desirable aroma, color and flavor (Yaylayan, 2005; Yaylayan & Keyhani, 1999), the Maillard reaction can also form undesirable advanced glycation end products with potential mutagenic, cytotoxic or carcinogenic effects, such as acrylamide (ACR) and 5-hydroxymethylfurfural/HMF (Nguyen et al., 2016). Acrylamide forms in the presence of Asn and reducing sugars at baking, roasting and frying temperatures at or above 120 °C (De Vleeschouwer et al., 2006). Low pH could increase the formation of HMF, even at low temperatures (Ummay Mahfuza, Md, Nadia, Md. Ibrahim, & Siew Hua, 2018). Since the soft pretzels in this research were baked at 218°C for 14 min, using sunflower flour, glucose, and sucrose, the formation of Maillard reaction intermediates such as Schiff bases and α -dicarbonyls, low, and high molecular weight melanoidins is promoted.

2.10 FTIR analysis of functional groups and protein structure modifications in sunflower flour mixtures

Fourier transform infrared spectroscopy (FTIR) is widely used for identifying functional groups in a sample using a set of standard spectra to compare with. FTIR measures the ability of a sample to absorb light in the mid-IR spectrum (4000-650 cm^{-1}) (Vandevoort, 1992). Although HPLC and LC-MS are used for the separation and analysis of CGA and MRP contents, N. J.

Liang et al. (2016) suggested FTIR as an alternative to HPLC as FTIR is more efficient when used in combination with the Attenuated Total Reflectance (ATR) technique and accessory, making it easier for sample preparation and handling (Vandervoort, 1992). When analyzing samples using ATR-FTIR, solid samples can be ground and placed directly on the ATR accessory for analysis. Columns, solvents, or highly trained individuals are not needed when using ATR-FTIR. N. J. Liang et al. (2016) used ATR-FTIR spectroscopy for the rapid identification of CGA and its isomers in pulverized green coffee beans. Rein & Rodriguez-Saona (2013) used portable FTIR spectrometry to analyze the ACR content in 64 different potato chip samples.

Oxidized chlorogenic acid quinones are highly reactive and react with proteins by covalent bonding, leading to the formation of C-N and C-S bonds between the nucleophilic regions on proteins (i.e., ϵ -amine group of lysine, sulfhydryl group of cysteine and terminal amino groups on polypeptide chains) and phenols. This addition reaction leads to structural modifications in proteins due to the binding of phenols with secondary structures of proteins, which was monitored using FTIR.

Although using HPLC for quantification is more complicated as the sample preparation and analysis readings are comparatively more time-consuming, it is the preferred method when analyzing samples with a complex matrix comprising of various compounds as they cannot be separated and may interfere with the FTIR analysis. Therefore HPLC would be the recommended method when analyzing complex matrices as it separates components of the mixture (Li & Brown, 2003).

2.11 Chlorogenic acid and protein content differences in sunflower flours and formation of MRPs

Chlorogenic acid is a reactant in both the Maillard browning and the greening reaction (S. Liang et al., 2018). Cai et al. (2014) found that adding 0.5 $\mu\text{mol/ml}$ CGA to Asn/glucose mixture increased ACR formation when compared with the control. Chlorogenic acid in its unoxidized non-quinone form increases formation of ACR by promoting HMF formation (Cai et al., 2014) whereas its *o*-quinone derivative decreased ACR as *o*-quinones react with amino acids, decreasing the probability of amino acids that could react with HMF, hence decreasing the production of ACR (Jiang et al., 2013).

A summary of how various treatments or ingredients affect the formation of CGA-quinone induced greening (TBA and CGA), and Maillard browning reactants and products (Schiff bases, ACR, and HMF) is provided in **Table 2**.

Table 2. Effects of different treatments and ingredients on CGA-quinone induced greening and Maillard browning

Variables Analyzed	Method of Analysis	Results	Hypotheses	Reference
Sugar Type effect on ACR and HMF formation	HPLC and LC-MS/MS: ACR HPLC and UV: HMF	HMF was highest in biscuits with glucose and fructose, while Acrylamide was highest in biscuits with glucose and the highest Asn concentration.	Mixtures prepared with glucose will contribute to formation of MRPs more than samples prepared using non-reducing sugars, such as sucralose.	(Nguyen et al., 2016)
Acidic ingredients effect on (phenols, CGA, protein), Trp and Schiff Base	HPLC: CGA, TBA, Caffeic Acid TEAC and Folin-Ciocalteu: Scavenging ABTS• FI using Spectrofluorometry: Trp, Schiff Base	<p>pH was positively correlated to greening and negatively correlated to CGA content.</p> <p>Using Maple Syrup increased pH, % green, and water activity.</p> <p>Honey lowered pH, % green, and water activity.</p>	The higher the pH, the higher formation of green TBA derivatives, and the lower concentration of free CGA in the mixtures.	(S. Liang et al., 2018)
Liquid sweeteners and relative humidity effect on CGA and TBA, protein	Spectrophotometer: Protein HPLC and UV-Vis: CGA and TBA LC-MS with ESI-MS: Adducts	<p>Maple: highest greening due to higher moisture</p> <p>CGA-lysine adducts, lowest free CGA.</p> <p>High moisture and high pH promoted greening.</p>	Higher moisture promote greening in pretzels and mixtures, but as the moisture content decreases, the formation of MRPs will decrease.	(S. H. Liang & Were, 2018b)
Independent variable: CGA Dependent variables: ACR formation and 3-	LC-MS/MS: ACR LC-MS with Abs: HMF UPLC-ESI/MS: 3-APA	<p>CGA addition to Asn/Glucose MR system increased ACR formation.</p> <p>Quinone derivatives decreased ACR formation,</p>	The higher free CGA content in the samples will promote formation of MRPs such as ACR and 3-	(Cai et al., 2014)

APA formation		by forming HMF.	APA since CGA is a precursor to the Maillard reaction.
Dependent variables: and their effect on ACR and HMF formation- 4 types of wheat flour with different fructose and glucose to Asn ratios	LC-ESI-MS: Asn, ACR, HMF	Asn + fructose formed Schiff base, bypassing Amadori products to produce ACR. Fructose contributed to ACR and HMF formation in all 4 flour types.	(Nguyen, van der Fels-Klerx, & van Boekel, 2017)
Egg replacers and baking temperatures on formation of TBA derivatives	LC-MS: TBA UV-Vis Spec: Schiff Base	Most greening occurred with highest moisture content and lowest concentration of antioxidants. Egg with lower temperature had highest moisture, internal greening, and TBA derivative formation.	(Rogers et al., 2018)

2.12 Rationale and Significance

Sunflower protein can function as an alternative to soy protein in gluten-free baked foods as it can provide similar functionalities, such as gelling, water-holding, thickening, emulsification, and binding. Sunflower protein can also be used to develop a high protein gluten-free, soft-baked pretzel. The global plant protein market is expected to grow by 8% CAGR by 2021. Six sources of proteins, one being sunflower, were identified as having the potential to be the next leading alternative proteins used for human consumption (Givaudan & Berkeley, 2019). However, the hydroxyl group on the phenol CGA present in HF 45 and HF 55 SFF, can covalently bind to proteins, decreasing the protein bioavailability and digestibility as well as its aforementioned functionalities in food (Alexandrino, Ferrari, de Oliveira, Ormenese, & Pacheco, 2017; Alu'datt, Rababah, Ereifej, Brewer, & Alli, 2013; Karefyllakis et al., 2018; Wildermuth et al., 2016). The benefit of retaining phenolics such as CGA, is due to their potential antioxidant capacity (Wildermuth et al., 2016). Although CGA interacts with food components such as proteins, carbohydrates, and lipids, further investigation is required to determine the effect of these interactions in functionality in food applications.

The overall goal was to investigate the effect of CGA from the sunflower flours containing 45 and 55% protein on CGA-quinone induced greening and MRPs, using model systems (flour mixtures) and gluten-free pretzels. This overall goal was accomplished through the following aims:

- I) Determine whether the CGA in the sunflower flour is contributing more to the formation of MRPs or TBA derivatives. It was hypothesized that chlorogenic acid would contribute more to the formation of green TBA derivatives as greening begins without the addition of heat to the system. In the presence of heat and

alkaline pH, CGA-quinone enhances Maillard browning and formation of Schiff bases, but once the heat is removed, the formation of MRPs would slow down (Domínguez, Núñez, & Lema, 1995; Gonzalez-Perez et al., 2002; Ozdal, Capanoglu, & Altay, 2013).

- II) Elucidate the effect of partially replacing gluten-free flour with Heliaflor[®] 45 and Heliaflor[®] 55 (45 and 55% protein content, respectively) at different levels (replacing 3.65 and 7.30% GF flour with SFF), using different sweeteners (reducing: glucose and non-reducing: sucrose and sorbitol), on the formation of Schiff bases, melanoidins, and green TBA derivatives. It was hypothesized that replacing gluten free flour with Heliaflor[®] 45 will result in an increased CGA content, which under alkaline pH will oxidize to form green TBA derivatives at a higher concentration than MRPs. Additionally, once heat is removed, MRP formation will either decrease or stop; however, CGA-quinone induced greening will continue. Lastly, the presence of sorbitol and sucrose will not contribute as much to either color-forming reaction, as much as glucose.
- III) Develop a naturally green gluten-free soft pretzel using sunflower flour that does not develop an undesirable color. It was hypothesized that replacing GF flour blend with 3.65% Heliaflor[®] 55 instead of 7.30% would provide a sufficient amount of CGA for green color formation, resulting in green pretzels. Heliaflor[®] 55 was expected to have a lower CGA and higher protein content. Organic solvents to produce sunflower protein leads to a sunflower isolate with increased protein content and decreased phenolic content (Wildermuth et al., 2016). To promote greening in pretzels, it was hypothesized that using Heliaflor[®] 45 at

7.30% will produce pretzels with increased greening.

By understanding how different quantities of CGA in two different sunflower flours used in combination with three different sweeteners (reducing and non-reducing) contribute as reactants for the Maillard, and CGA-quinone-induced greening reactions, a gluten-free soft pretzel with added protein and natural green color can be developed.

Research involving the application of sunflower flour in various baked goods, especially those which are more alkaline, where the development of CGA-quinone induced greening is promoted is lacking. When Yue, Hettiarachchy, & Dappolonia (1991) incorporated native and succinylated sunflower protein concentrate and isolate to wheat flour in bread baking, the succinylated sunflower protein had detrimental effects on the bread (grey, dense crumb with rounded corners and edges). Succinylation of the sunflower protein may have broken the covalent linkages, denaturing the protein's helical structure that would have otherwise contributed to elasticity in baking. However, breads prepared using the flour blends containing 5% native sunflower protein concentrate and isolate produced acceptable loaves, whereas the crumb structure was no longer stable and fell apart at higher concentrations (10 and 15%). Ashraf & Siandwazi (1986) found that the maximum level at which wheat flour could be replaced with sunflower protein concentrate in gingerbread cookie dough was 30%. Using this formula, (Ashraf & Siandwazi, 1986) produced cookies supplemented with protein content double that of cookies baked with 0% sunflower protein concentrate (7.2 versus 15.9% protein). At a 30% replacement, they did not observe any detrimental effects to the baking process, physical and sensory characteristics of the cookies.

This research will expand the knowledge of using SFF in alkaline foods, in addition to pretzels. Producing a gluten-free soft baked pretzel that contains sunflower protein to their menu,

bakeries, and restaurants can cater to the 1.5 million U.S.A consumers allergic to soy (Gupta et al., 2019), those with Celiac's disease or those that are gluten-free diets due to gluten sensitivity. Additionally, the knowledge and experience of developing a pretzel containing sunflower flour and protein can be applicable when preparing a variety of both gluten-free and conventional foods, including but not limited to breads, cakes, noodles, cookies, biscuits, pancakes, and muffins, as proposed by Planetarians (2019). Consumers with dietary restrictions due to Celiac's disease or IBS as a result of gluten intolerance can benefit from a gluten-free alternative, and the baked goods industry can benefit from profits from the sales.

3. MATERIALS AND METHODS

3.1 Ingredient procurement for formulating pretzels using sunflower flours

Two 2.7 kg samples of organic sunflower flours: Heliaflor[®] 45 and Heliaflor[®] 55 were obtained from Austrade Inc. (Palm Beach Gardens, Fla., USA.). Xanthan gum was obtained from Jungbunzlauer Inc. (Des Plaines, IL., USA.), while the remaining ingredients: Bob's Red Mill[®] gluten free 1:1 baking flour/ UPC: 039978014535, Morton[®] iodized salt/ UPC: 024600011013, Arm & Hammer[®] pure baking soda/ UPC: 033200974031, Fleischmann's[®] instant yeast/ UPC: 040100009282, Wholesome[™] organic vegan cane sugar/ UPC: 012511405007, Kroger[®] baking powder/ UPC: 01111090765, Lekithos[®] sunflower lecithin/ UPC: 85755400148, Kroger[®] unsalted butter/ UPC: 01111089305 and Simple Truth Organic[™] cage free, large brown eggs/ UPC: 1111079771 were purchased from a local grocery store.

Chlorogenic acid (98%) standard, HPLC grade acetonitrile, and HPLC water were purchased from Thermo Fisher Scientific (Hampton, NH). A Luna Omega 5 μ C18 (2) 100 Å (150 $\times$ 2 mm, 1.5 μ m particle size) column was purchased from Phenomenex[®] (Torrance, CA).

3.2 Experimental Design

Each dough formed five pretzels. A total of five pretzel formulations, including the control, were duplicated. A pretzel from each formulation was crumbled using a mortar and pestle, and a sample from each treatment was obtained to analyze the Schiff base, CGA, melanoidins, and green TBA derivatives formed from baking soft pretzels as a result of the content of protein in two different sunflower flours (Heliaflor[®] 45 [$\geq 41\%$ protein] and Heliaflor[®] 55 [$\geq 51\%$ protein]). The concentrations used were (0.5%, 3.65%, 5.50%, 7.30% and 10.5% replacement in gluten-free [GF] dough) and the processing conditions were controlled for each formulation (**Figure 5**). The Schiff base, CGA, melanoidins and green TBA derivatives were quantified using UV-Vis or HPLC as shown in **Figure 6**.

The development of greening and browning as a result of the protein type, concentration used, and storage time (2 hours post-baking and 18 hours post-baking, stored at room temperature) was analyzed using the Hunter L, a, b colorimeter.

Figure 5. Experimental Design and analysis for gluten-free pretzels made using SFF 45 and 55

Figure 6. Sample preparation and summary of variables tested, their methods of analysis and hypothesis of outcomes

3.3 Formulation of gluten-free pretzels using sunflower flours

The pretzels were made using a gluten-free recipe developed to optimize the texture and flavor comparable with pretzel made using wheat flour. **Figure 5** provides ingredient percentages of the five formulations used to make the pretzels: the control with 0% SFF, the other four modifications were replacing 4.29 g (3.65%) and 8.58 g (7.30%) of the GF flour with sunflower flours - Heliaflor[®] 45 (HF 45) and replacing 4.29 g (3.65%) and 8.58 g (7.30%) of the GF flour with Heliaflor[®] 55 (HF 55). The dough (261.55 g total) was formed using a Kitchen Aid mixer with the whisk attachment. Once the dough was prepared, the bowls containing the doughs were covered with plastic wrap and left to proof at ambient temperature for 1 h. After an hour, the dough was portioned into 50 g balls, stretched, and the pretzels were shaped using a Brentwood TS-251 Non-Stick Mini Pretzel Maker Machine, to control the weight and shape of samples for analysis. The shaped pretzels were proofed covered for an additional 30 min. They were then dipped in a 1.2% baking soda/boiling water bath for 30 s and placed on a perforated tray for an additional 30 s to remove any dripping water. The pretzels were then baked in a convection oven (Maytag Model MGR8800FZ, MI) for 14 min at 218 °C to ensure even heat distribution and quick baking.

3.4 Proximate analysis of sunflower flours and pretzels, using vacuum drying and Soxhlet extraction

Pretzels were removed from the -80°C freezer and thawed at room temperature for 4 hours before grinding and weighing onto aluminum weighing pans. Samples were dried in a vacuum oven for 24 hours, with temperature and vacuum set to 60 °C and -25 in.-Hg., respectively. The dehydrated pretzels were Soxhlet extracted for lipid content determination and further used for HPLC and UV-Vis analysis (section 3.5.3).

3.5 Physical analysis of pretzels developed using sunflower flours (Heliaflor® 45 or 55) at 0, 3.65, and 7.30%, and gluten-free flour blend

3.5.1 pH of pretzels

The pH of 0.5 g baked pretzels with 25 mL HPLC water mixtures was measured as outlined in AACCI Method 02-52.01 (AACC, 1999; International, Approved November 3, 1999.; S. H. Liang & Were, 2018a). The samples were homogenized for 1 min at 1.3×10^3 g, using the Multi-prep Homogenizer (PRO Scientific Inc., Oxford, CT. USA). The mixtures were incubated for 15 min before centrifugation (Thermo ST8 Centrifuge, Thermo Fisher Scientific, Inc. CA. USA) at 9×10^3 g at 4°C for 30 min. The pH of the supernatant obtained was measured at 10-minute intervals, over 30 minutes using a pH electrode attached to a LabQuest data collection system (Vernier Software & Technology, OR. USA).

3.5.2 Hunter L, a, b color analysis

Pretzels were stored ambient in a closed glass cupboard post-baking, in order to mimic the storage conditions of baked goods at a grocery store or bakery. A Hunter *L, a, b* spectrophotometer (CM-2500D, Konica Minolta, Inc. Tokyo, Japan) was used to measure the *L** [lightness (100) to darkness (0)], *a** [redness (+a) and greenness (-a)] and *b** [yellowness (+b) and blueness (-b)] of the external surface and internal cross-section of the pretzels. A white plate was used to calibrate the spectrophotometer. Pretzels were cut cross-sectionally to measure internal color. Initial measurements were taken 2-hours post-baking. The remaining pretzels were left stored ambient in cabinets and evaluated 18 hours post-baking for changes in color. Pretzels that were not used for Hunter *L, a, b* color analysis were stored at - 80°C for further analysis. All samples were analyzed in triplicates.

3.5.3 Identification of CGA-quinone induced greening and Maillard browning reactants and end products in defatted pretzels using UV-Vis

Defatted pretzel (0.5 g) prepared by Soxhlet fat extraction was homogenized with deionized water (5.0 mL) for 1 min at 1.3×10^3 g, using the Multi-prep Homogenizer (PRO Scientific Inc., Oxford, CT. USA). The mixtures were then centrifuged at 5×10^3 g (4696 rpm) at 4 °C for 30 minutes. The supernatant obtained was further centrifuged at 9×10^3 g at 4 °C for 15 min to minimize turbidity. The samples were filtered through a 0.45 µm syringe filter and diluted 1:100 using DI water. The diluted pretzel mixtures were transferred to a clear 96-well Corning microplate for UV-Vis analysis using the FLUOstar Omega Microplate Reader (BMG Labtech, Ortenberg, Germany). Schiff bases, CGA, melanoidins, and green TBA derivatives were measured at 294, 320, 420 and 680 nm, respectively.

3.6 Kjeldahl protein quantification of gluten-free flour blend, 45 and 55% sunflower flours

The protein of the GF flour blend and the two sunflower flours (HF 45 and HF 55) were quantified using Kjeldahl as outlined by Chang & Zhang (2017). **Table 3** includes the percentages for moisture, lipid, and protein in the GF flour blend and the two sunflower flours (SFFs). The pretzels were not analyzed for their protein content using Kjeldahl.

Table 3. Proximate analysis of Heliaflor® 45, Heliaflor® 55, and GF flour blend (%)

Sample	Moisture	Lipid Content	Protein	Protein**	Carbohydrate (by difference)***
Gluten Free Flour Blend	8.62 [†]	1.16 [†]	5.14 [†]	5.78	85.08
Heliaflor® 45	4.38*	max. 13*	≥ 41*	44.93	41.62
Heliaflor® 55	4.20*	max. 2.5*	≥ 51*	50.75	42.3

*Based on product specification sheet provided by Austrade Inc. (Palm Beach Gardens, Fla., USA.)

[†]Based on product specification sheet provided by Bob's Red Mill Natural Foods, Inc.® (Milwaukee, OR., USA.)

**Based on Kjeldahl analysis

***Values are estimated base on moisture, fat and protein content (ash was not included)

3.7 Model mixture preparation for HPLC and UV-Vis analysis

Flour mixtures prepared in triplicates using Heliaflor[®] 45, 55, and GF flour blend (abbreviated to HF 45, HF 55, and GF, respectively), are shown in **Table 4**.

Table 4. Model mixtures prepared with gluten-free flour, Heliaflor[®] 45, Heliaflor[®] 55 with glucose

Sample Number	Treatment	Quantities* (g)
1	Heliaflor [®] HF 45	5.0
2	Heliaflor [®] HF 55	5.0
3	GF**	5.0
4	Replacing 0.5% GF with Heliaflor [®] 45	0.003 HF 45 + 4.98 GF
5	Replacing 3.65% GF with Heliaflor [®] 45	0.18 HF 45 + 4.82 GF
6	Replacing 5.50% GF with Heliaflor [®] 45	0.28 HF 45 + 4.73 GF
7	Replacing 7.30% GF with Heliaflor [®] 45	0.37 HF 45 + 4.64 GF
8	Replacing 10.50% GF with Heliaflor [®] 45	0.53 HF 45 + 4.48 GF
9	Replacing 0.5% GF with Heliaflor [®] 55	0.003 HF 55 + 4.98 GF
10	Replacing 3.65% GF with Heliaflor [®] 55	0.18 HF 55 + 4.82 GF
11	Replacing 5.50% GF with Heliaflor [®] 55	0.28 HF 55 + 4.73 GF
12	Replacing 7.30% GF with Heliaflor [®] 55	0.37 HF 55 + 4.64 GF
13	Replacing 10.50% GF with Heliaflor [®] 55	0.53 HF 55 + 4.48 GF

* 40 mL DI water and 0.75 g baking soda was added to all treatments.

**GF is a 1:1 gluten-free flour baking blend purchased from Bob's Red Mill (contains sweet rice flour, whole grain brown rice flour, potato starch, whole grain sorghum flour, tapioca flour, xanthan gum)

Samples 14-26 were prepared using the same quantities of Heliaflor[®]45, Heliaflor[®]55, and GF flour as samples 1–13, with an additional 0.25 g glucose (0.5%) in each sample. All ingredients were weighed, vortexed, and homogenized with 40 mL DI water for 1 min at 1.08 x 10⁴g. The pH of the mixtures was measured and adjusted to 8.04 - 8.12 using baking soda. The sample tubes were incubated at 94 °C for 13 minutes in a Precision SWB 27 Shaking Water Bath (Thermo Fisher Scientific, Waltham, MA) at 55 rpm. Once cooled, the mixtures were further centrifuged, and samples were prepared using the same method as that used for UV-Vis analysis of defatted pretzels (section 3.5.3) with one modification – the filtered supernatant obtain from flour mixtures were diluted 1:10, instead of 1:100 as shown in **Figure 7**.

Figure 7. Schiff bases, melanoidins, CGA, and green compounds Analysis

3.8 Chlorogenic acid and green trihydroxybenzacridine derivatives quantification

Samples for HPLC analysis of CGA and green TBA derivatives were prepared using the same method as those for UV-Vis analysis and diluted 1:5 (Error! Reference source not found.).

An Agilent 1100 series HPLC (Agilent Technologies, Inc. Santa Clara, CA, USA) with a G1315B diode array detector (DAD), was used for monitoring compounds at 294 nm, 320 nm, 420 nm, and 680 nm, using a Phenomenex® Luna Omega 5µ C18 (2) 100 Å (150 × 2 mm, 1.5 µm particle size) column. Mobile phases A and B were comprised of HPLC water acidified with 0.1% (v/v) formic acid and acetonitrile acidified with 0.1% (v/v) formic acid, respectively. The

gradient used was 0 min, 6% B; 2 min, 7.0% B; 2.5 min, 7.2% B; 3 min, 7.30% B; 4 min, 7.4% B at 0.8 mL/min flow rate, oven temperature controlled at 30°C, injection volume set to 20.0 µL. Chlorogenic acid was diluted to 2.5, 5, 7.5, 10, 15 and 20% using HPLC-grade water, and external standard curve at 320nm (**Figure 8**) was used to calculate CGA in the flour mixtures.

Figure 8. CGA Standard curve prepared at different concentrations (2.5, 5.0, 7.5, 10.0, 15.0, and 20.0%)

3.9 Identification of CGA and changes in protein molecular structures in flour mixtures using Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy

Flour mixtures for FTIR analysis were prepared using only SFF 55 instead of both SFF 45 and 55, as there was no significant difference in the formation of CGA-quinone induced greening or MRPs (Schiff bases and melanoidins) between mixtures containing HF 45 and 55 when analyzed using UV-Vis. Mixtures with various concentrations of sunflower flour (replacing 0, 0.5, 3.65, 5.5, 7.30, and 10.50% GF with HF 55) were used to replace the GF flour blend and combined with three different sweeteners (glucose, sucrose or sorbitol), with quantities listed in **Table 5**. Samples 8-14 and 15-21 were prepared using the same ingredients and quantities as samples 1-7, with the exception that sorbitol was replaced with glucose and sucrose,

respectively. The flour mixtures for FTIR analysis were prepared similar to the samples for HPLC and UV-Vis analysis. After incubating the samples for 13 minutes at 94°C, the samples were vortexed to break the gel that forms as a result of the starches and flours (potato starch, tapioca starch, rice flours) that comprise the gluten-free flour blend, being hydrated and heated. Approximately 3.5g of each sample was then transferred to an aluminum weigh boat and vacuum dried at 41°C, at -22 in.-Hg. The samples were then transferred, covered, to a -20°C freezer for a week, before analysis.

Table 5. Dehydrated Model Mixtures with Sorbitol, Glucose, and Sucrose Prepared for FTIR Analysis

Sample*	Concentration of GF Flour (g)	Concentration of HF 55 (g)
Samples Prepared with Sorbitol		
1	5	0
2	4.975	0.003
3	4.818	0.183
4	4.725	0.275
5	4.635	0.365
6	4.475	0.525
7	0	5
Samples Prepared with Glucose		
8	5	0
9	4.975	0.003
10	4.818	0.183
11	4.725	0.275
12	4.635	0.365
13	4.475	0.525
14	0	5
Samples Prepared with Sucrose		
15	5	0
16	4.975	0.003
17	4.818	0.183
18	4.725	0.275
19	4.635	0.365
20	4.475	0.525
21	0	5

*All samples contained 40 mL DI water, 0.75g baking soda, and 0.25g sweetener (sorbitol, glucose, or sucrose)

Identification of CGA and other compounds present in the flour mixtures, as a result of their bonds' bending and stretching in the fingerprint region, was done using the Shimadzu IRAffinity-1S Fourier Transform Infrared Spectrometer (Shimadzu, Kyoto, Japan) with the ATR accessory. The spectrometer was set in the absorbance mode, and spectra were collected over a wavenumber range of 4000-700 cm^{-1} at 2 cm^{-1} spectral resolution, with 16 scans for each spectral collection. A background scan was obtained before sample scans and before every 3rd sample thereafter.

3.10 Statistical Analysis

The effects of SFF concentration (3.65 versus 7.30% replacement of GF flour), types of sunflower flour (HF 45 versus 55) and storage time post-baking (2 versus 18 hours) on the formation of CGA-quinone induced greening and Maillard browning on the surface and internal cross-section in soft baked pretzels was analyzed using four-way ANOVA and Duncan's multiple range test. The effects of SFF concentration (0, 0.5, 3.65, 5.5, 7.30, 10.5% replacement of GF flour), storage time post-sample preparation (1 hour versus 18 hours) and sweetener type (sorbitol, glucose, and sucrose) on the formation of MRPs was analyzed using a three-way ANOVA and Duncan's multiple range test. Correlations between amount, type of sunflower flour, type of sweetener, storage time, concentration of CGA, CGA-quinone induced greening, and Maillard browning was analyzed using Statistical Analysis Software 9.3 (SAS Institute Inc. NC, USA), where results are considered significant if $P < 0.05$ throughout.

4. RESULTS AND DISCUSSION

4.1 Moisture and fat contents of gluten-free pretzels developed using sunflower flours comprising 45 and 55% protein (HF 45 and 55)

There was no significant difference in the moisture content of the five pretzel formulations since the ingredients that contribute moisture to the pretzels were used at the same concentrations in all formulations **Table 6**.

Table 6. Moisture and fat contents of gluten-free soft pretzels prepared using Heliaflor® 45 and Heliaflor® 55 at 3.65 and 7.30%

Treatment	Moisture (% w.b.)	Fat (% d.b.)
Control	33.67 ± 0.04 ^a	1.75 ± 0.008 ^a
Heliaflor® 45 (3.65% replacement)	32.95 ± 0.06 ^a	2.96 ± 0.008 ^b
Heliaflor® 45 (7.30% replacement)	31.70 ± 0.09 ^a	2.78 ± 0.001 ^b
Heliaflor® 55 (3.65% replacement)	34.69 ± 0.07 ^a	4.10 ± 0.027 ^c
Heliaflor® 55 (7.30% replacement)	35.00 ± 0.35 ^a	2.78 ± 0.003 ^b

Protein analysis on the pretzels was not carried out.

^{a,b,c} Means within a column with different superscript letters differ ($P < 0.05$)

The pretzels made by replacing 3.65% of the GF blend with HF 55, however, had higher fat (4.10%) than the pretzels prepared without any SFF (control: 1.75% fat) and pretzels made by replacing 7.30% GF blend with H55 (2.78% fat). Based on the product specification sheet from Austrade Inc., and the CO₂ flush step used to obtain the lower fat-containing sunflower flour, the lipid content of HF 55 sunflower flour is lower than that of HF 45 (2.5% and 13%, respectively). The 4.10% higher fat in the pretzel prepared with HF 55 at 3.65% is potentially due to the difference in the amount of butter applied to coat the pretzels post-baking as the butter was not weighed before coating.

4.2 Protein content of pretzels prepared with and without sunflower flours

The protein contents of the pretzels determined using the ESHA Research nutrition analysis software (Salem, OR) (control, addition of SFF 45 or 55 at 3.65 or 7.30%) is shown in **Table 7**. Based on the generated nutrition facts panels, pretzels prepared with Heliaflor® 55 at

7.30% had the highest protein content (8g/100g serving) and the control, which did not contain any sunflower flour, had the lowest protein content (4g/100g serving). Using sunflower flour with the higher protein (55 versus 45%) at a higher concentration in the pretzels increased the protein content in the pretzels. Therefore, the pretzels prepared using HF 55 at 7.30% would be recommended to someone who is avoiding gluten but is interested in getting the highest possible protein by consuming pretzels with SFF.

The manufacturing process by which Heliaflor[®] 55 is produced includes CO₂ extraction to decrease fat and increase the protein content (**Figure 2**). However, the total fat content of pretzels prepared using HF 45 was 3.5 g versus 3 g in pretzels prepared using HF 55, based on the Nutrition panel obtained from ESHA. This difference was not significant.

The results obtained from the Soxhlet analysis of pretzels (**Table 6**) are not supported by those generated using ESHA (**Table 7**). Using the ESHA software is more accurate for nutritional data based on the actual formulations. However, based on the results obtained from Soxhlet analysis, the difference in fat content between pretzels prepared by replacing GF with 3.65% H45 (2.96% fat) versus 3.65% H55 (4.10% fat) is due to human error.

Table 7. Nutrition fact panels generated using ESHA for pretzels prepared using Heliaflor® 45 and 55 at 3.65 or 7.30% (per 100 g serving size)

Treatment				
Control	3.65% Heliaflor® 45	7.30% Heliaflor® 45	3.65% Heliaflor® 55	7.30% Heliaflor® 55
Nutrition Facts Serving Size (100g) Servings Per Container <hr/> Amount Per Serving Calories 240 Calories from Fat 25 <hr/> % Daily Value* Total Fat 3g 5% Saturated Fat 1.5g 8% Trans Fat 0g Cholesterol 5mg 2% Sodium 850mg 35% Total Carbohydrate 47g 16% Dietary Fiber 2g 8% Sugars 5g Protein 4g <hr/> Vitamin A 2% • Vitamin C 0% Calcium 2% • Iron 2%	Nutrition Facts Serving Size (100g) Servings Per Container <hr/> Amount Per Serving Calories 230 Calories from Fat 30 <hr/> % Daily Value* Total Fat 3.5g 5% Saturated Fat 1.5g 8% Trans Fat 0g Cholesterol 5mg 2% Sodium 860mg 36% Total Carbohydrate 44g 15% Dietary Fiber 4g 16% Sugars 5g Protein 6g <hr/> Vitamin A 2% • Vitamin C 0% Calcium 2% • Iron 4%	Nutrition Facts Serving Size (100g) Servings Per Container <hr/> Amount Per Serving Calories 230 Calories from Fat 35 <hr/> % Daily Value* Total Fat 3.5g 5% Saturated Fat 1.5g 8% Trans Fat 0g Cholesterol 5mg 2% Sodium 860mg 36% Total Carbohydrate 43g 14% Dietary Fiber 5g 20% Sugars 5g Protein 7g <hr/> Vitamin A 2% • Vitamin C 0% Calcium 2% • Iron 4%	Nutrition Facts Serving Size (100g) Servings Per Container <hr/> Amount Per Serving Calories 230 Calories from Fat 25 <hr/> % Daily Value* Total Fat 3g 5% Saturated Fat 1.5g 8% Trans Fat 0g Cholesterol 5mg 2% Sodium 860mg 36% Total Carbohydrate 44g 15% Dietary Fiber 2g 8% Sugars 5g Protein 6g <hr/> Vitamin A 2% • Vitamin C 0% Calcium 2% • Iron 4%	Nutrition Facts Serving Size (100g) Servings Per Container <hr/> Amount Per Serving Calories 230 Calories from Fat 25 <hr/> % Daily Value* Total Fat 3g 5% Saturated Fat 1.5g 8% Trans Fat 0g Cholesterol 5mg 2% Sodium 860mg 36% Total Carbohydrate 43g 14% Dietary Fiber 2g 8% Sugars 5g Protein 8g <hr/> Vitamin A 2% • Vitamin C 0% Calcium 4% • Iron 4%

4.3 Color development in gluten-free pretzels developed using sunflower flours comprised of 45 and 55% protein (HF 45 and 55), based on Hunter L, a, b

The kneaded pretzel doughs containing SFF developed greening during proofing, before being shaped into pretzels. The dough surface that was exposed to air was green within 30 minutes after kneading versus the bottom of the dough that was in contact with the surface of the bowl, where there was no greening until after baking. In the presence of oxygen and a basic environment as a result of using alkaline ingredients such as egg whites and baking soda, CGA is oxidized to CGA-quinone in doughs containing sunflower flour, which further reacts with amino groups and forms green TBA derivatives. If dough is not exposed to the air, and does not have as much access to oxygen from the environment; the greening occurs at a slower rate (S. H. Liang & Were, 2018a; Wildermuth et al., 2016).

Gluten-free pretzels were initially formulated to contain 10 and 20% sunflower (HF 45 or 55) flour (**Figure 9**). As expected, the pretzel prepared without any SFF did not green (**Figure 9a**), as dough contained no CGA in the formulation.

Figure 9 Initial iterations of gluten-free soft pretzels prepared with and without HF 45 - control without HF (a), GF flour + 3.65% HF 45 (b), and GF flour + 10% HF 45 (c)

However, pretzels baked using 10% HF 45 developed an unappealing dark green-black color (**Figure 9c**). Using HF 45 at 3.65% produced pretzels with a visually more appealing brown-green color (**Figure 9b**). Therefore, the pretzels were reformulated with decreased quantities of SFF (**Table 8**).

Decreasing the concentrations of SFF used to replace GF flour from 10 and 20% to 3.65 and 7.30% produced pretzels with a better visual appearance. The green color that developed after CGA-quinone oxidation, resulting from the different concentrations of SFF used (3.65 and 7.30%), were greener than the color of the pretzel in **Figure 9c** (replacing GF flour with 10% HF 45).

As expected, pretzels prepared without any SFF did not green and only browned as a result of Maillard browning. As the SFF content increased from 3.65 to 7.30%, the greening also increased due to the higher CGA content in the sunflower flours. The surface and internal cross-section Hunter *L*, *a*, *b* values of the pretzels, measured 2- and 18-hours post-baking shown in **Table 8** indicate that the surface of the pretzels was lighter than the internal cross-sections, 2- and 18- hours after baking. There was a 12.05 - 26.91% decrease in surface *L* values after 18 hours, indicating increased darkening in all the pretzels, except the control where the *L* value did not change. The surface of the pretzels remained darker than the internal cross-section 18-hours post storage. The *L* values of the internal cross-section did not change.

There were some differences in internal *L* values, however, the differences were not perceptible. The *L* values of the pretzels' surface decreased more after 18 hours than that of the inner cross-section, indicating that over time, the surface darkened more than the internal cross-section of the pretzels due to slower continual Maillard browning and CGA-quinone induced greening, as a result of higher surface temperature during baking and exposure to oxygen from the environment over time, respectively (S. Liang et al., 2018; Rogers et al., 2018; Wildermuth et al., 2016).

Table 8. Progression of internal and surface greening in pretzels as a function of protein content in sunflower flour, concentration and time during ambient storage

2 Hours Post-Baking				
Control	HF 45 at 3.65%	HF 45 at 7.30%	HF 55 at 3.65%	HF 55 at 7.30%
<u>Surface color</u>				
Hunter $L^*70.20 \pm 1.50^c$ $a^*4.15 \pm 0.85^d$ $b^*35.41 \pm 0.44^a$	Hunter $L^*66.65 \pm 0.18^c$ $a^*-1.65 \pm 0.28^c$ $b^*39.21 \pm 0.68^b$	Hunter $L^*59.83 \pm 3.12^d$ $a^*0.28 \pm 1.22^c$ $b^*39.66 \pm 1.22^b$	Hunter $L^*66.43 \pm 3.23^c$ $a^*4.86 \pm 1.20^d$ $b^*45.95 \pm 0.64^c$	Hunter $L^*56.90 \pm 0.93^d$ $a^*-4.05 \pm 2.94^e$ $b^*35.33 \pm 2.24^a$

Surface Internal				
<u>Internal color</u>				
Hunter $L^*43.88 \pm 2.81^b$ $a^*18.44 \pm 1.45^a$ $b^*26.47 \pm 1.42^a$	Hunter $L^*48.81 \pm 2.97^a$ $a^*17.31 \pm 0.13^a$ $b^*33.09 \pm 3.62^a$	Hunter $L^*39.02 \pm 5.27^b$ $a^*12.91 \pm 2.62^b$ $b^*25.79 \pm 11.39^a$	Hunter $L^*37.37 \pm 0.98^b$ $a^*17.12 \pm 1.02^a$ $b^*19.51 \pm 1.06^a$	Hunter $L^*42.52 \pm 4.47^b$ $a^*12.42 \pm 9.08^a$ $b^*26.35 \pm 4.40^a$
18 Hours Post-Baking				
<u>Surface color</u>				
Control Hunter $L^*70.82 \pm 0.97^e$ $a^*4.19 \pm 1.03^e$ $b^*36.97 \pm 3.04^c$	HF 45 at 3.65% Hunter $L^*58.62 \pm 1.35^d$ $a^*-7.38 \pm 0.65^c$ $b^*29.57 \pm 0.65^a$	HF 45 at 7.30% Hunter $L^*43.73 \pm 2.41^a$ $a^*-7.64 \pm 0.08^c$ $b^*25.58 \pm 2.29^b$	HF 55 at 3.65% Hunter $L^*58.07 \pm 2.16^d$ $a^*1.90 \pm 1.05^b$ $b^*36.18 \pm 1.03^c$	HF 55 at 7.30% Hunter $L^*46.12 \pm 3.18^a$ $a^*-10.64 \pm 0.62^c$ $b^*21.68 \pm 0.19^b$

Hunter $L^*44.53 \pm 1.84^a$ $a^*17.27 \pm 0.20^d$ $b^*30.01 \pm 0.85^a$	Hunter $L^*49.27 \pm 0.82^a$ $a^*12.21 \pm 1.44^a$ $b^*31.34 \pm 5.48^a$	Hunter $L^*36.83 \pm 3.38^b$ $a^*11.39 \pm 2.49^a$ $b^*28.03 \pm 2.76^a$	Hunter $L^*34.00 \pm 4.13^b$ $a^*14.61 \pm 2.18^a$ $b^*15.16 \pm 7.90^b$	Hunter $L^*42.64 \pm 2.75^a$ $a^*2.78 \pm 2.81^b$ $b^*23.33 \pm 0.42^b$

^{a,b,c,d,e} Means within a row with different superscript letters differ ($P < 0.05$)

Heating can accelerate the greening reaction (Wildermuth et al., 2016). Additionally, CGA from the SFF, alkaline pH, and oxygen, especially on the surface, promoted greening over time. Pretzels prepared using HF 55 to replace 7.30% GF had the highest greening 2 and 18 hours post-baking (-4.05 and -10.64, respectively), which is not in line with the initial hypothesis. Since Helialfor® 45 had 6% lower protein content (44.93%) than Helialfor® 55 (50.75%), it was expected that HF 45 would have more CGA than that in HF 55 because in commercial sunflower protein extraction process, organic aqueous solvents or membrane filtration technology are used to remove the phenolics which effect the color of the proteins, however, HF 45 and 55 are not produced using organic solvents and rather rely on processing using CO₂ to extract the lipids and preserve the polyphenols from the sunflower meal.

The higher the protein in concentrate or isolate, typically the lower its phenolic (CGA) content compared to the flour (Wildermuth et al., 2016). However, the methods by which Austrade Inc. produces the SFFs retain phenolics, as heat and O₂ are absent which prevents the oxidation of CGA-quinone. Pretzels prepared using HF 55 had higher greening than those prepared using HF 45 due to the higher phenolic content retained using CO₂ extraction. Heating accelerates greening, and as hypothesized, the *a* values of all pretzels made using the sunflower flours decreased significantly after 18 hours because the CGA-quinone induced greening reaction does not require heat. The *b* values for the pretzels decreased over time, indicating decreased yellowness in the pretzel.

As shown in **Figure 10**, the Hunter *L*, *a*, *b* values for all pretzels generally decreased 18 hours after baking, except for the controls made without any SFF. The surface of the pretzels prepared using HF 55 at 7.30% had the highest greening (lowest *a* value), and the second-to-last darkest *L*-value of 46.12 after 18 hours. Pretzels prepared using HF 45 at 7.30% had the lowest

L-value: 43.73. The internal greening of the pretzels did not change significantly depending on the type of protein used (HF 45 versus HF 55), except in the pretzel prepared using HF 55 at 7.30%, which had the highest greening (*a*-value: 2.78) 18 h post-baking, due to the highest concentration of CGA from the sunflower flour. It was expected that the cross-sections of the pretzels would be moister than the surface if the internal and surface pH were the same, leading to increased CGA-quinone induced greening internally (Rogers et al., 2018). However, this was not supported by the pretzels' Hunter L, a, b results because the surface of the pretzel has a higher pH than the internal cross-section from dipping the pretzels in the soda-water before baking. The alkaline surface pH contributed more to the CGA-quinone induced greening than the moisture content inside the pretzels.

Figure 10. Changes in L, a, b values, 2- and 18-hours post-baking, in pretzels prepared without sunflower flour (Control), replacing gluten-free (GF) flour with Helialflor® 3.65 (HF 45 3.65%) and Helialflor® 7.30% (HF 45 7.30%) SFF containing 45% protein (HF 45) and replacing GF flour with 3.65 (HF 55 3.65%) and 7.30% (HF 55 7.30%) SFF containing 55% protein (HF 55).

The treatment, time, and position (internal versus surface of pretzels) individually had significant ($P<0.05$) effects on CGA-quinone induced greening in the pretzels **Table 9**. The % protein content in the HF 45 versus 55 sunflower flours was not different as the two flours' protein contents only differed by 5.81%. However, the interaction between SFF type (HF 45

versus 55) and SFF concentration used (Flour: treatment) was significant ($P < 0.0001$). The SFF concentration (Treatment) and the time post-baking combination had a significant effect ($P = 0.0086$) on the greening in the pretzels as well as the three-way interaction between protein type, the concentration of sunflower flour used, and time post-baking.

Table 9. Four-way model analysis of variance of greening (a*) values

Independent variables	Degrees of Freedom	Mean Square	P-value
Treatment^a	2	348.411851	<0.0001
Flour type	1	0.462169	0.7901
Time^c	1	278.727707	<0.0001
Position^d	1	3485.693040	<0.0001
Flour:Treatment	1	222.181102	<0.0001
Treatment:Time	2	34.537245	0.0086
Flour:Time	1	0.390602	0.8067
Treatment:Position	2	4.923453	0.4721
Flour:Position	1	44.064169	0.0125
Time:Position	1	1.568167	0.6243
Flour:Treatment:Position	1	26.210852	0.0504
Flour:Treatment:Time	1	27.709602	0.0445
Treatment:Time:Position	2	2.073006	0.7265
Flour:Time:Position	1	17.412252	0.1079
Flour:Treatment:Time:Position	1	16.066102	0.1220

^aTreatment includes protein concentrations: 0, 3.65 or 7.30% GF flour replacement

^bFlour type includes HF 45 or HF 55

^cTime includes 2- or 18- hours post-baking

^dPosition includes internal cross-section or external surface

Greening increased over time due to the unreacted CGA in the SFF, which continued to react over time. The internal and surface pH of the pretzels and oxidation due to the presence of oxygen, since the pretzels were stored in an ambient room in closed cabinets on flat trays, to mimic grocery stores, contributed to the increased greening over time.

4.4 Schiff bases, chlorogenic acid, melanoidins and green trihydroxybenzacrindine derivatives in defatted pretzels

Green TBA derivatives developed in all pretzels made using SFF, but there was no greening in the control since the control did not contain any SFF (**Figure 11d**). The pretzel formulated by replacing 7.30% GF with HF 55 had the highest Schiff bases (**Figure 11a**), melanoidins (**Figure 11c**) and green TBA (**Figure 11d**) absorbance as a result of highest CGA concentration (**Figure 11b**), owing to the highest SFF protein content used to prepare the pretzels.

Figure 11 Schiff bases (a), chlorogenic acid (b), melanoidins (c), and green trihydroxy benzacrindine derivatives (d) in pretzels made with and without Heliator® 45 and 55 (HF 45 and 55).

*SFP = sunflower protein (HF 45 or HF 55)

Pretzels made using HF 45 at 3.65% had the lowest Schiff bases and CGA (**Figure 11a** and **Figure 11b**) due to their lower concentration of SFF. The control pretzel did not have any green TBA derivatives (**Figure 11d**), but absorbed at 320 nm (**Figure 11b**), which may be due to the presence of other non-CGA hydroxycinnamic acids (HCA's) such as caffeic, 3,5-diferuloylquinic acid and their ester derivatives, and ferulic acid in brown rice, sorghum, and white rice flours, 5-caffeoylquinic acid in white rice flour, and *p*-coumaric acid in sorghum and white rice flours of the GF flour blend (Rocchetti et al., 2017; Schroter et al., 2018), as there is no sunflower flour in the control pretzels.

4.5 Chlorogenic acid, melanoidins and green trihydroxybenzacridine derivatives in flour mixtures prepared using sunflower flour, gluten-free flour blend, $\pm$ glucose

The CGA content in pretzels made by replacing GF flour with HF 45 or 55 at 3.65 or 7.30% was not significantly different. Heliaflor[®] 55, which had 5.81% more protein than HF 45, was expected to have a lower CGA concentration, which was not supported by the UV-Vis or HPLC quantification of the pretzels (**Figure 11**). Based on the UV-Vis analysis, using either HF 45 or 55 SFF at 3.65% is feasible for developing a naturally green gluten-free pretzels.

Model mixtures prepared using different concentrations of HF 45 and 55 with and without glucose were analyzed 2- and 18-hours post-incubation. As shown in **Figure 12**, adding glucose or using HF 45 versus HF 55 did not significantly affect the formation of brown and green compounds. In samples analyzed at 0 hours (**Figure 12a** and **b**), there were higher brown than green pigments. After 18 hours, the concentration of green pigments increased, whereas the content of brown pigments did not change significantly (**Figure 12c** and **d**).

- (a) Flour mixtures prepared using GF flour, HF 45 or HF 55, with and without glucose monitored for formation melanoidins immediately after incubation

- (b) Flour mixtures prepared using GF flour, HF 45 or HF 55, with and without glucose monitored for formation of green TBA derivatives immediately after incubation

- (c) Flour mixtures prepared using GF flour, HF 45 or HF 55, with and without glucose monitored for formation of melanoidins 18 hours after incubation

- (d) Flour mixtures prepared using GF flour, HF 45 or HF 55, with and without glucose monitored for formation of green TBA derivatives 18 hours after incubation

Figure 12 Concentration of brown (A_{410}) compounds formed in flour mixtures at 0 hours (a) and 18 hours (c) and green (A_{680}) compounds formed at 0 hours (b) and 18 hours (d), in flour mixtures measured prepared by replacing GF flour with HF 45 or HF 55 at 0, 3.65, 5.5, 7.30, 10.50 and 100%, using UV-Vis.

The Maillard reaction occurs due to the condensation between reducing sugars and amino groups in a thermal environment, in this case, baking. Once there was no more change in temperature, the formation of brown pigments decreased. However, since the measured pH of the pretzels is alkaline (pH 8.04 – 8.12), the reaction between the free CGA and amino acids from the sunflower flour continues over time, increasing the formation of green pigments. Although heat accelerates the reaction, temperature is not crucial for CGA-quinone induced greening reaction in an alkaline environment. However, temperature is a more critical factor affecting the Maillard browning reaction, as it speeds up the browning and greening reactions (Rogers et al., 2018; Wildermuth et al., 2016).

Baking soda (for pH adjustment)+HF 45 or 55 SFF's (without GF flour) +water mixtures when incubated at 94 °C had the highest green (A_{680}) and brown (A_{420}) compounds in comparison to treatments without HF 45 or 55, due to free CGA coming from the two sunflower flours. From three-way ANOVA on the effect of protein type (HF 45 or HF 55), concentration and presence or absence of glucose from the flour mixtures (**Table 10**), the protein concentration was the only significant variable ($P<0.001$) affecting formation of Schiff bases, CGA, melanoidins, and green TBA derivatives in the flour mixtures.

The presence or absence of glucose in the flour mixtures did not significantly affect Schiff bases ($P=0.2394$) or melanoidins ($P=0.2819$) formation, as carbonyls such as CGA in the sunflower flours contribute to the formation of CGA-quinone induced greening and formation of MRPs more than the presence or absence of a reducing sugar (S. H. Liang & Were, 2018b). This could be due to the low (0.5%) concentration of glucose that was added to the mixtures. Altering the glucose content added to the flour mixtures may have a significant effect, where the higher the concentration of glucose added, the higher the Schiff base and melanoidin formation.

Table 10. Three-way model analysis of variance of Schiff bases, chlorogenic acid, melanoidins, and green trihydroxybenzacridine derivatives

Independent variables		Schiff bases (A294nm)	Chlorogenic Acid (A320nm)	Melanoidins (A420nm)	TBA Derivatives (A680nm)
Treatment (Percent SFF) ^a	df	6	6	6	6
	ms	54.1071417	32.5115901	2.24105765	1.14130330
	P	<.0001	<0.0001	<.0001	<.0001
Flour (HF 45 or HF 55) ^b	df	1	1	1	1
	ms	0.1221564	0.2234575	0.01155344	0.01336781
	P	0.6247	0.4199	0.4752	0.5399
± Glucose ^c	df	1	1	1	1
	ms	0.7148984	0.6451775	0.02641488	0.01098647
	P	0.2394	0.1731	0.2819	0.5783
Interaction (flour*treatment)	df	5	5	5	5
	ms	0.0836736	0.1002816	0.00762238	0.00634184
	P	0.9739	0.9124	0.8853	0.9686
Interaction (treatment*glucose)	df	6	6	6	6
	ms	0.3132609	0.2545082	0.00994184	0.00202016
	P	0.7123	0.6097	0.8448	0.9992
Interaction (flour*glucose)	df	1	1	1	1
	ms	0.1397724	0.0886003	0.01126799	0.00096068
	P	0.6009	0.6108	0.4807	0.8692
Interaction (flour*treatment*glucose)	df	5	5	5	5
	ms	0.0260468	0.0263448	0.00388948	0.00631770
	P	0.9982	0.9953	0.9710	0.9689

^aTreatment (Percent SFF) includes protein concentrations: 0, 0.5, 3.65, 5.5, 7.30, 10.50 or 100% GF flour replacement

^bFlour (HF 45 or HF 55) includes the percent protein in the HF 45 or HF 55 flours (45 vs. 55%)

^cGlucose includes the presence or absence of glucose from the flour mixtures

*df refers to degrees of freedom; ms refers to mean square; P refers to p value

Since there was no significant difference in the Schiff bases or melanoidins when using HF 45 versus 55 (protein type), the model mixtures with the different sweeteners (sorbitol, glucose, or sucrose) were prepared only using different quantities of HF 55 (HF 45 was not a variable). Three-way ANOVA (**Table 11**) indicated that the concentration of HF 55, time, sweetener type, individually and the interactions between storage time and protein concentration, protein concentration and sweetener type, storage time, sweetener type and storage time, sweetener type and protein concentration all had a significant effect ($P<0.0001$) on the formation of Schiff bases, melanoidins and green TBA derivatives and the CGA content.

Table 11. Three-way model analysis of variance of Schiff bases, chlorogenic acid, melanoidins, and green trihydroxybenzacridine derivatives

Independent variables		Schiff bases (A _{294nm})	Chlorogenic Acid (A _{320nm})	Melanoidins (A _{420nm})	TBA Derivatives (A _{680nm})
Treatment ^a	df	6	6	6	6
	ms	13.3547582	19.5993295	21.6834980	8.98987573
	P	<.0001	<0.0001	<0.0001	<0.0001
Time ^b	df	1	1	1	1
	ms	0.09941619	0.462169	1.3396986	15.28708714
	P	<.0001	<0.0001	<0.0001	<0.0001
Sweetener ^c	df	2	2	2	2
	ms	4.23239329	3.9200995	1.0023113	0.10736646
	P	<0.0001	<0.0001	<0.0001	<0.0001
Interaction (time*treatment)	df	6	6	6	6
	ms	0.01175746	0.0393013	0.0524731	1.71095502
	P	<0.0001	<0.0001	<0.0001	<0.0001
Interaction (treatment*sweetener)	df	11	11	11	11
	ms	0.69624113	0.3784486	0.2909893	0.74142211
	P	<0.0001	<0.0001	<0.0001	<0.0001
Interaction (time*sweetener)	df	2	2	2	2
	ms	0.01250148	0.0085796	0.0526283	0.15366939
	P	<.0001	<0.0001	<0.0001	<0.0001
Interaction (time*treatment*sweetener)	df	11	11	11	11
	ms	0.01045700	0.0238870	0.0170154	0.09844596
	P	<.0001	<0.0001	<0.0001	<0.0001

^aTreatment includes protein concentrations: 0, 0.5, 3.65, 5.5, 7.30, 10.50 or 100% GF flour replacement

^bTime includes 0- or 18- hours post-incubation

^cSweetener includes the sorbitol, glucose or sucrose

*df refers to degrees of freedom; ms refers to mean square; P refers to p value

The three-way ANOVA support the hypothesis that using a higher concentration of SFF and increased storage time increases the concentration of MRPs and CGA-quinone induced greening in pretzels (**Figure 11**) and flour mixtures (**Table 11**) prepared using SFF. Adding glucose did not impact the formation of MRPs and CGA-quinone induced greening based on the three-way ANOVA shown in **Table 10**, possibly due to the low concentration that was used. However, the presence of reducing sugars (glucose) increased Schiff bases (S. H. Liang & Were, 2018a), which supports the results in **Table 11**, where the sweetener type used has a significant effect ($P<0.0001$) on formation of Schiff bases and melanoidins as a result of the Maillard and

CGA-quinone induced greening reactions. As time (0- to 18- hours post-incubation) and concentration of protein increases, the CGA-quinone induced greening increases, and the formation of MRPs only increases slightly.

Although HPLC analysis can quantify CGA in the baked pretzels, pretzels prepared using the two different types of sunflower flours (HF 45 and 55) at the three different concentrations (0, 3.65 and 7.30%) were not quantified for CGA using HPLC. However, CGA content in flour mixtures containing different concentrations of HF 45 and 55, with and without glucose, were quantified using HPLC.

4.6 Free chlorogenic acid in flour mixtures prepared with sunflower flour, gluten-free flour, and glucose

As expected, increasing concentration of SFF in mixtures correlated positively to the peaks due to the increased concentration of CGA from the SFF as Flour mixtures prepared with H45 by replacing 10.50% of the GF flour had the highest CGA concentration (**Figure 13**). The mixture prepared using only GF flour (0% SFF) should not contain any CGA as there is no sunflower flour, however, the peak can be from HCA's present in the GF flour which also absorb at 320 nm.

The mixture prepared using 10.50% HF 45 (**Figure 13**) had the highest peak at 320 nm (0.0656 mM CGA), whereas the mixture prepared using H55 at 0.50% had the lowest peak height. As the amount of HF 45 increased in the flour mixtures from 3.65 to 10.50%, the unreacted CGA also increased (**Figure 14**), supporting the hypothesis that using a higher concentration of SFF would increase CGA and therefore increase formation of green TBA derivatives in the high pH baked pretzels.

Figure 13. HPLC chromatographs for mixtures prepared by replacing 3.65, 5.50, 7.30, and 10.50% GF with Heliaflor® 45 (H45) and 0.50% Heliaflor® 55 (H55).

The mixture prepared without any SFF (0% SFF/GF Control) (**Figure 13**) also had peak absorption at 320 nm, which may be due to the presence of other hydroxycinnamic acids (HCA's) such as caffeic, ferulic, *p*-coumaric acids and their ester derivatives (Rocchetti et al., 2017; Schroter et al., 2018) present in the gluten-free flour blend or the SFF, which also absorb at 320 nm (Shrestha et al., 2017).

Figure 14. Free chlorogenic acid (mM) in flour mixtures prepared without sunflower protein (GF 0%), using Heliaflor® 45 and 55 (HF 45 and 55) at the same concentrations in the flour mixtures (0.4, 0.6, 0.8, 1.7 and 10.92%), measured using HPLC

When added to the mixture, glucose reacts with proteins in the flour to produce Maillard browning. The CGA from the SFF can compete with the glucose, when present at higher concentrations than glucose, and react with protein to produce MRP Schiff bases, melanoidins, and CGA-quinone induced greening products. The increase in both the brown and green color in pretzels prepared using SFF supported the hypothesis that adding sunflower flour to the GF flour blend will increase the formation of green and brown color due to the addition of CGA from the sunflower flour to the mixture (S. H. Liang & Were, 2018a; Rogers et al., 2018).

Three-way ANOVA indicated that the concentration of SFF ($P < 0.0001$) and addition or exclusion of glucose ($P = 0.0259$) were the only significant factors affecting the CGA content in flour mixtures prepared with and without glucose (**Table 12**).

Table 12. Three-way model analysis of variance of chlorogenic acid (CGA) or hydroxycinnamic acid (HCA) content as effected by protein type (HF 45 or 55), the concentration used in the presence or absence of glucose

Independent variables		A ₃₂₀
Treatment ^a	Df	6
	Ms	0.24785020
	P	<.0001
Protein (type) ^b	Df	1
	Ms	0.00627383
	P	0.2051
Glucose ^c	Df	1
	Ms	0.02008153
	P	0.0259
Interaction (protein*treatment)	Df	5
	Ms	0.00797068
	P	0.0819
Interaction (treatment*glucose)	Df	6
	Ms	0.00561315
	P	0.2063
Interaction (protein*glucose)	Df	1
	Ms	0.00192582
	P	0.4802
Interaction (protein*treatment*glucose)	Df	5
	Ms	0.00293754
	P	0.5745

^aTreatment includes protein concentrations: 0, 0.5, 3.65, 5.5, 7.30, 10.50 or 100% GF flour replacement

^bProtein (type) includes HF 45 or HF 55 (45 versus 55% protein content in SFF)

^cGlucose includes the presence or absence of glucose from the flour mixtures

*df refers to degrees of freedom; ms refers to mean square; P refers to p value

Using reducing or non-reducing sweeteners in the flour mixtures did not have a significant effect on the CGA content in the flour mixtures. It was initially hypothesized that mixtures prepared using glucose and sucrose would have the highest concentration of CGA-induced green TBA derivatives, since the CGA would only contribute to the greening reaction where it does not have to compete as a carbonyl, with reducing sugars in Maillard browning. Van der Fels-Klerx et al. (2014) found that crystalline sucrose needs to be melted prior to being hydrolyzed. Sucrose melted at approximately 180 °C after 10-11 minutes of baking in biscuits,

leading to increased development of ACR and HMF in biscuits. Karel & Labuza (1968) found that non-enzymatic browning occurred in solutions comprising sucrose and citric acid at low moisture contents, due to acidic hydrolysis of sucrose. The CGA content was expected to be higher in mixtures containing reducing sugars as CGA is only contributing to one reaction (CGA-quinone induced greening) instead of two (greening and Maillard browning).

As shown in **Figure 15**, flour mixtures that did not contain any SFF had the highest CGA concentration analyzed using HPLC due to the unreacted HCA's from the GF flour blend.

Figure 15. Chlorogenic acid (CGA) (mM) in flour mixtures prepared using sunflower flour containing 55% protein (HF 55) at different concentrations in combination with various sweeteners (sorbitol, glucose or sucrose), quantified using HPLC

Mixtures containing glucose and sucrose followed a similar trend, wherein the concentration of free CGA decreased when low concentrations of HF 55 were present in the mixtures (0.183 g) and as the concentration of HF 55 increased from 0.183 to 0.275 g, the CGA content increased and decreased again when 0.365g of HF 55. This may be due to CGA being used up as a reactant in the Maillard browning and CGA-quinone induced greening reactions. The free CGA remained constant when HF 55 concentration was increased from 0.365 to 0.525

g. However, flour mixtures containing sorbitol did not follow the same trend as mixtures prepared using glucose and sucrose, as the lowest concentration of unreacted CGA was found in sorbitol mixtures prepared with 0.275 g HF 55, and the highest content of free CGA was found in sorbitol mixtures prepared with 0.003 g HF 55 followed by 0.365 g HF 55. Since sorbitol is a non-reducing sugar, it is not a reactant in Maillard browning. Therefore, the free CGA from SFF in flour mixtures prepared with sorbitol may have contributed to CGA-quinone induced greening and was not a component in the formation of Schiff bases and Maillard browning. Hence, the decrease in free CGA as HF 55 content increased from 0 to 0.275 g. In all mixtures prepared with sorbitol, glucose, and sucrose, the CGA from SFF contributed to both CGA-quinone induced greening and Maillard browning, therefore when used at lower concentrations, it was almost immediately used up in both reactions, especially when present in mixtures with glucose, which is a readily available reducing sugar.

4.7 Fourier transform infrared spectrometry of compounds developed from Maillard browning and CGA-quinone induced greening in flour mixtures, using

There were no significant differences in the CGA, Schiff base, melanoidins, and green TBA derivatives in the flour mixtures prepared using HF 45 versus 55 when analyzed with HPLC and UV-Vis. Therefore, FTIR analysis was done only on the flour mixtures with HF 55. The FTIR spectra of the HF 55 flour mixtures prepared using three different sweeteners (sorbitol, glucose, and sucrose)+ baking soda+ HF 55 at 0, 0.01, 0.40, 0.60, 0.79, 1.14, and 10.87%, to replace GF flour are shown in **Figure 16**. All samples prepared for FTIR analysis were dehydrated as water in the samples can interfere with peaks of other adsorbed organic molecules in samples being analyzed (Lefevre, Preocanin, & Lutzenkirchen, 2012). Owing to its high polarity, OH stretching, and H bond vibrations, peaks from water can be seen in the range of

1640-3300 cm^{-1} (Manley, Van Zyl, & Osborne, 2002). This may cause water to interfere in peaks of other OH-containing compounds, such as phenols. Therefore, flour mixtures were dehydrated for a week in a vacuum oven to ensure complete dehydration prior to analysis.

Fourier transform infrared spectrometry monitors structural changes that occur in secondary (α -helices and β -sheets) structures of proteins. The respective secondary structure forms based on the sequence of hydrogen bonding between the amine hydrogen and carbonyl oxygen, resulting in the peptide backbone of proteins (Karefyllakis et al., 2018). According to Terrile et al. (2016), the following regions can be used to identify CGA using FTIR: 1452, 1380, 1260, 1128, and 1059 cm^{-1} . Of the three spectra prepared using different sweeteners shown in **Figure 16 a, b, and c**, most mixtures have the highest absorbance peak at 1032 cm^{-1} , owing to higher CGA, similar to findings by N. J. Liang et al. (2016) in green coffee beans. Isomers of CGA have a strong infrared absorption spectrum in the range of 1300-800 cm^{-1} . Amongst all the flour mixtures prepared using sorbitol (**Figure 16a**), the mixture prepared by replacing 100% of the GF flour with SFF 55 had the highest absorbance at 1032 cm^{-1} , as expected due to the highest concentration of SFF in the mixture, resulting in the highest peak. However, the same trend did not apply to flour mixtures with glucose (**Figure 16b**) and sucrose (**Figure 16c**). Flour mixtures prepared without any sunflower flour (0% HF 55 replacement) did not absorb or have peaks throughout the spectra because there may not have been any structural conformations due to the lack of SFF in the mixtures.

The fingerprint region of the FTIR spectra between 1400-500 cm^{-1} (Nallamuthu, Devi, & Khanum, 2015), and peaks in this region are a result of bending vibrations. The functional group region of the FTIR spectra ranges from 1400-4000 cm^{-1} , and this region contains few peaks as a result of stretching due to the infrared radiation absorbed by the bonds. Carbohydrates contain

various C-C, C-O and O-H bonds which undergo C-C and C-O bending between 1500-500 cm^{-1} and O-H stretching at 3500-3000 cm^{-1} (Terrile et al., 2016).

The infrared spectra of proteins is comprised of various amide bands due to the vibrations from peptide moieties, generally associated with the secondary structure of proteins. The amide I band (C=O stretching) ranges from 1600-1700 cm^{-1} , and the amide II band ranges from 1500-1600 cm^{-1} (C-N stretching coupled with N-H bending) (Karefyllakis et al., 2018). Peaks in the amide I and amide II regions are associated with α -helices. All the flour mixtures had peaks within the 1500-1600 and 1600-1700 cm^{-1} regions, with mixtures containing sucrose having the highest peaks. Peaks in this region are generally due to the stretching vibrations of the double bonds in alkenes (C=C) and carbonyls (C=O) (Yi et al., 2019).

Sucrose itself does not contain any C=C or C=O bonds, but its components glucose and fructose do. If the sucrose degrades to glucose and fructose monosaccharides above 120 °C (De Vleeschouwer et al., 2006), this leads to peaks in the 1500-2000 region due to C=O bond stretching from glucose and fructose. Mixtures prepared using sorbitol and glucose had the least peaks in 1500-1600 and 1600-1700 cm^{-1} region. Although flour mixtures prepared using glucose should have high peaks in 1500-1600 and 1600-1700 cm^{-1} regions due to the free carbonyls, there may be a shift to a higher frequency when glucose, a reducing sugar, reacts with amines from the SFF to generate MRPs (**Figure 16b**).

- a. Flour mixtures prepared using sorbitol, GF flour and different concentrations of HF 55

- b. Flour mixtures prepared using glucose, GF flour and different concentrations of HF 55

- c. Flour mixtures prepared using sucrose, GF flour and different concentrations of HF 55

Figure 16. FTIR spectra of flour mixtures prepared using sorbitol (a), glucose (b), and sucrose (c) by replacing GF flour with HF 55 at 0, 0.50, 3.65, 5.50, 7.30, 10.50, and 100% in the mixtures. The dotted line represents absorption at 1032 cm⁻¹

As the concentration of SFF used increased in flour mixtures prepared using sucrose, the peak absorbance also increased, except for mixtures wherein GF was replaced with 100% SFF, as there were no peaks in the 800-1800 cm^{-1} region. The absence of peaks may be due to decrease in α -helical structures leading to a slightly deformed, less helical structure, due to conformational changes from the binding of CGA with proteins in the matrix. Replacing 100% GF with SFF may contribute more to the formation of CGA-quinone-protein adducts instead of using SFF at lower concentrations. Therefore, samples that do not contain SFF should not have any changes in helical protein's secondary structure, whereas as the concentration of SFF increases in the mixtures, the CGA content increases, which should increase the helical structure conformations of the proteins owing to the increased polyphenol-protein complex formation.

Other than mixtures containing sorbitol, the flour mixtures prepared using glucose and sucrose did not follow the expected trend, where an increase in SFF used to replace GF also had higher peaks in the FTIR analysis. Flour mixtures without any HF 55 did not have any peaks throughout the spectrum. The lack of peaks in a FTIR spectrum can be due to the molecules in the compound having symmetrical bonds and, therefore no dipole moment. Hence, these bonds are not affected by the infrared radiation that is applied to them, and there is no bond stretching or bending.

The ATR-FTIR spectra shown in **Figure 17-Figure 19** Show that majority of the mixtures have a distinct peak at approximately 1000 cm^{-1} , which can be due to CGA or other non-CGA HCA's in the mixtures (1032 cm^{-1}) similar to those in **Figure 16**. Peaks in this region can also be due to the S=O stretching from sulfoxides, such as those found in cysteine in SFF or CO-O-CO stretching from anhydrides.

a. Sample 1 – 0% HF 55

b. Sample 2 – 0.50% HF 55

c. Sample 3 – 3.65% HF 55

d. Sample 4 – 5.5% HF 55

e. Sample 5 – 7.30% HF 55

f. Sample 6 – 10.50% HF 55

g. Sample 7 – 100% HF 55

Figure 17. Flour mixtures prepared with sorbitol and replacing GF flour blend with 0 (a), 0.5 (b), 3.65 (c), 5.50 (d), 7.30 (e), 10.50 (f), and 100% (g) Heliator® 55

a. Sample 8 – 0% HF 55

b. Sample 9 – 0.5% HF 55

c. Sample 10 – 3.65% HF 55

d. Sample 11 – 5.50% HF 55

e. Sample 12 – 7.30% HF 55

f. Sample 13 – 10.50% HF 55

g. Sample 14 – 100% HF 55

Figure 18. Flour mixtures prepared with glucose and replacing GF flour blend with 0 (a), 0.5 (b), 3.65 (c), 5.50 (d), 7.30 (e), 10.50 (f), and 100% (g) Heliaplor® 55

a. Sample 15 - 0% HF 55

b. Sample 16 – 0.50% HF 55

c. Sample 17 – 3.65% HF 55

d. Sample 18 – 5.50% HF 55

e. Sample 19 – 7.30% HF 55

f. Sample 20 – 10.50% HF 55

g. Sample 21 – 100% HF 55

Figure 19. Flour mixtures prepared with sucrose and replacing GF flour blend with 0 (a), 0.5 (b), 3.65 (c), 5.50 (d), 7.30 (e), 10.50 (f), and 100% (g) Heliaflor® 55

All spectra in **Figure 17 - Figure 19** contain lots of peaks in the 1400 – 1700 cm^{-1} region, making it difficult to depict the structures that are present or that are forming as a result of the compounds in the mixtures' matrices and the processing (effect of heat on secondary protein structure). It was expected that the interaction between oxidized CGA-quinone and proteins, which would result in green TBA derivatives would alter the structure of the proteins, which can be monitored using FTIR.

The IR spectra of proteins comprises of amide bands. The secondary structures of proteins are determined based on the Amide I bands (C=O stretching) range from 1600-1700 cm^{-1} and Amide II bands (C-N stretching coupled with N-H bending) range from 1500-1600 cm^{-1} (Karefyllakis et al., 2018). The peaks within the Amide I and Amide II regions are associated with α -helices (peaks found in 1657-1651 cm^{-1} are associated with α -helices and peaks are in 1634-1608 cm^{-1} are associated with β -sheets). Polyphenol complexation alters the secondary structures of proteins, leading to an increase in α -helices and β -sheets, and random coil reduction, which causes protein structural stabilization. The larger the polyphenol, with higher OH groups, the higher the order of complexation between proteins and polyphenols. The shifts of

the Amide I band to a lower wavenumber are indicative of polyphenolic interaction occurring. As the concentration of CGA increased in the flour mixtures, a shift in Amide I peaks was noticed in the FTIR graphs and there was an increase in intensity in Amide I and Amide II bands, owing to the complex formation between protein-phenol. As shown in **Figure 19**, as the concentration of SFF was increased from 0 to 10.50%, the intensity in the peaks from 1500-1600 and 1600-1700 cm^{-1} increased, indicating changes in the α -helix and β -sheets, which suggests that the increase in polyphenol concentration causes an increase in polyphenol-protein interaction, increasing the formation of CGA-quinone induced greening.

5. SUMMARY AND CONCLUSIONS

Although the two flours supplied from Austrade Inc. contained different amounts of protein (44.93 and 50.75%), this difference in protein content did not significantly affect the formation of chlorogenic-quinone induced greening or Maillard browning products. As the concentration of SFF used to produce soft-pretzels and flour mixtures increased from using no SFF (control) to replacing 10.50% GF flour with SFF, the content of CGA, MRPs and green TBA derivatives also increased, owing to the higher CGA content in SFF. Heliaflor[®] 55 (HF 55: 50.75% protein) had higher CGA than Heliaflor[®] 45 (HF 45: 44.93% protein), when monitored using UV-Vis and HPLC, due to the process used by Austrade Inc. to produce sunflower flour, in which the polyphenols are retained. Unlike other protein extraction processes where the polyphenols are removed to avoid the green coloration, using organic solvent to extract the proteins or membrane filtration, refuted the hypothesis that HF 55 would contain less free CGA as a reactant for CGA-quinone induced greening. Therefore, replacing GF flour with the highest concentration of SFF (7.30%) contributed to the most greening owing to the highest CGA content present in the pretzels. As time increases, the oxygen, availability of free CGA in the

matrix, and the alkalinity from egg whites and baking soda in the pretzels' matrix contribute to increase in CGA-quinone induced green TBA derivatives. The development of Maillard browning slows down once the pretzels are cooled after baking, but some Maillard browning continued to occur on the surface of the pretzel. This is because the pretzel formulation contains reducing sugars and proteins, and although thermal treatment accelerates the formation of MRPs, it is not needed. Pretzels prepared using the highest concentration of sunflower flour (replacing 7.30% GF flour with HF 55) developed the darkest surface and internal greening. Free CGA initially contributed to the Maillard browning, but once heat was removed, free CGA contributed more to CGA-quinone induced greening than Maillard browning.

6. RECOMMENDATIONS FOR FUTURE STUDIES

Suggestions for future research include performing visual and organoleptic sensory analysis on the gluten-free soft-baked pretzels prepared using HF 45 and HF 55 at 3.65%, to determine preference between the two iterations and likelihood to purchase to a green gluten-free soft-baked pretzel.

Additional recommendations include using LC-MS to monitor the dicarbonyls that form in the pretzels, to determine the effect of using higher concentrations of CGA (than those used in this study) from sunflower flour, on the reactants and products of the Maillard reaction. This information can also determine whether CGA contributes more to CGA-quinone induced greening or Maillard browning reactions in the pretzels. Monitoring and quantifying the formation of carcinogenic and neurotoxic HMF and ACR, the by-products of the Maillard reaction, using LC-MS and accordingly, develop strategies to mitigate their formation in the soft-baked gluten-free pretzels. This information can determine if the sunflower flour pretzels can be promoted to consumers as containing lower concentrations of harmful MRPs. Since ACR is on

Prop 65 warnings in California as a chemical “known to the state to cause cancer or reproductive toxicity (such as birth defects and other reproductive harm),” if the pretzels contain lower ACR than traditional pretzels, they can be promoted accordingly to those concerned with monitoring their daily ACR consumption.

Lastly, since HF 55 is prepared using a method wherein CGA and the protein are preserved in the final flour, it is suggested to test the functionality, solubility, and digestibility of sunflower protein. The sunflower protein may complex with free CGA rendering the protein insoluble, indigestible, and unable to serve its functional and nutritional purpose in pretzels. Kjeldahl analysis is recommended to cross-check the protein content of the baked pretzels against the calculated protein content on the Nutrition Facts panel generated using ESHA.

7. REFERENCES

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