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Color and Structural Modifications of Alkaline Extracted Sunflower Protein Concentrates and Isolates Using L-Cysteine and Glutathione

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**Color and Structural Modifications of Alkaline extracted Sunflower Protein concentrates
and isolates using L-Cysteine and Glutathione**

A Thesis by

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Submitted in partial fulfillment of the requirements for the degree of

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May 2021

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ABSTRACT

Color and Structural Modifications of Alkaline extracted Sunflower Protein concentrates and isolates using L-Cysteine and Glutathione

by Akira Kurniawan Ishii

Sunflower protein can be extracted at alkaline pH with sulfites or organic solvent de-phenolization of sunflower seed meals if greening is unwanted. This greening is promoted at alkaline pH when chlorogenic acid (CGA) oxidizes and reacts with free amine groups from amino acids, such as lysine. Thiol-containing dough conditioners: L-cysteine hydrochloride and reduced glutathione (GSH) were investigated as an alternative de-greening strategy to de-phenolization. Varying pH from 7 to 11 and concentration of 0.82 to 5.6 mM were randomly assigned by Response Surface Methodology (RSM) to evaluate the combined and additive greening inhibition effect of thiol and extraction pH. The powders with the highest greening were control (pH 8.93-9.3 and no added thiols) and pH 10.41 with 0.82 mM with thiols added. The powders with cysteine however had more browning than GSH. From RSM, the maximum greening inhibition was achieved with pH 8.71 and 4.23 mM cysteine and pH 8.51 and 3.78 mM GSH. Furthermore, fluorescence spectroscopy revealed that cysteine had a protective effect against alkaline denaturation, whereas GSH quenched fluorescence. Overall, incorporation of thiol ingredients can be an alternative de-greening strategy for sunflower protein. These results could lead to extensive use of sunflower protein applications in non-allergenic and non-GMO plant-based meat alternatives or bakery products where greening is unwanted.

Keywords: alkaline extraction, chlorogenic acid, sunflower protein, thiol, greening inhibition, protein unfolding, fluorescence quenching.

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LIST OF ABBREVIATIONS

CD – Circular Dichroism

CGA – chlorogenic acid

CYS – L-cysteine hydrochloride (PubChem CID 60960)

DTNB - 5,5'-Dithiobis(2-nitrobenzoic acid) (PubChem CID 6254)

FTIR- Fourier-transform infrared spectroscopy

GSH – glutathione (PubChem CID 124886)

HPLC – High performance liquid chromatography

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SPI - sunflower protein isolate

TBA - Trihydroxy benzacridine

Logic Model

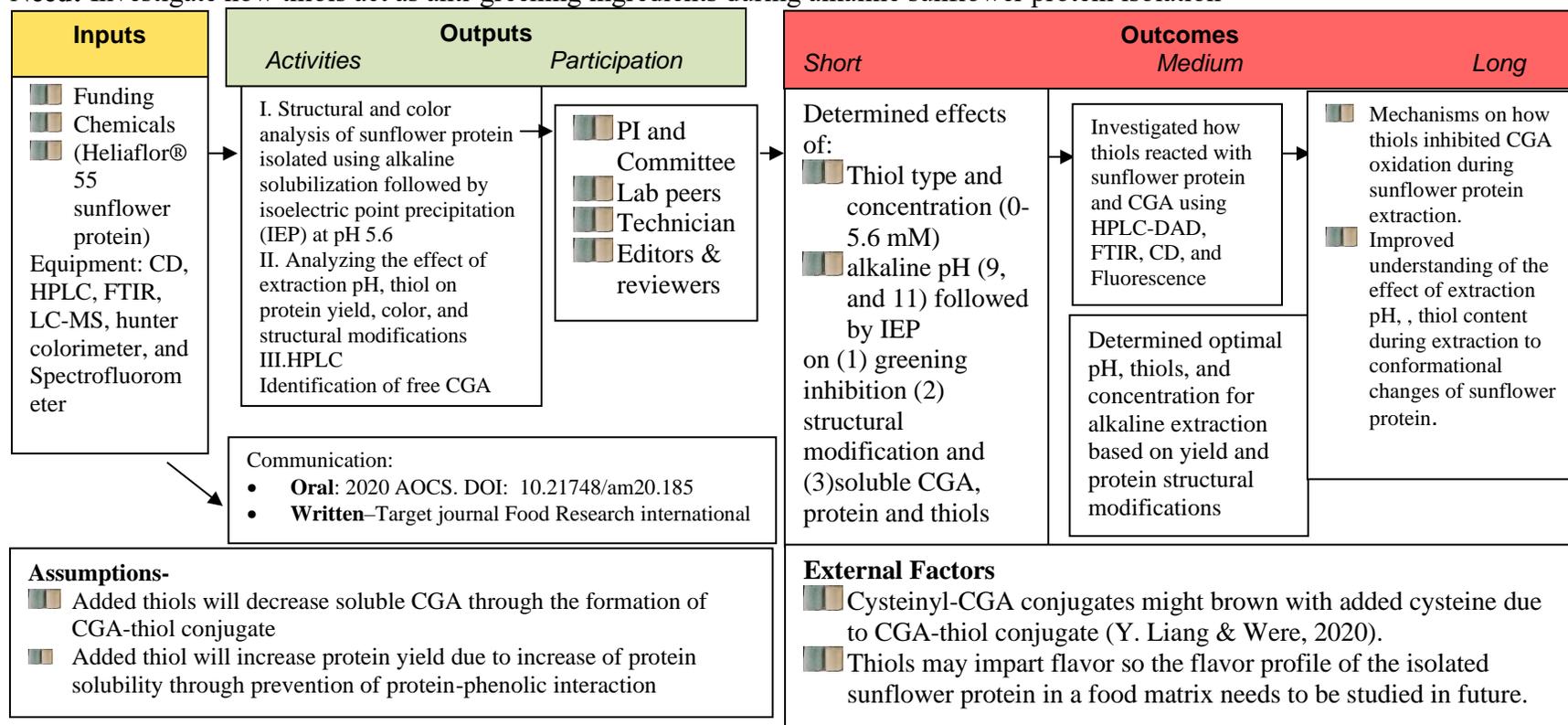
Program: Novel Foods and Innovative Manufacturing Technologies 2021 AFRI-NIFA Program Area Priority Codes A1364 –(USDA, 2021)

Situation:

During alkaline sunflower protein extraction, chlorogenic acid (CGA) oxidizes and conjugates with amino acids to form green trihydroxy benzacridine (TBA) and cause protein secondary structure modifications (Verena Bongartz et al., 2016; D. Karefyllakis, Salakou, Bitter, Goot, & Nikiforidis, 2018)

It was hypothesized that adding thiols during alkaline extraction would minimize greening as 1.4- 5.6 mM L-cysteine inhibited greening in CGA: Lysine solutions through reaction with oxidized CGA to form colorless thiol-CGA conjugates (Y. Liang & Were, 2020).

Need: Investigate how thiols act as anti-greening ingredients during alkaline sunflower protein isolation



1. Introduction

Sunflower protein is processed from sunflower seeds after sunflower oil extraction. As a protein source, sunflower protein has three times FAO's recommended daily values of sulfur-containing amino acids: methionine and cysteine (Albe Slabi, Mathé, et al., 2020; P. R. Salgado, Ortiz, Petrucci, & Mauri, 2012; WHO, 2006). In addition, sunflower protein is not categorized as a major food allergen in the United States (FDA, 2004), except for rare anaphylaxis reaction from roasted sunflower seeds (Alvarez, Mera, Baynova, Zavala, & Castaño, 2018). Thus, sunflower protein could be used to formulate plant-based vegan bakery products to achieve organic, allergen-free status and high-sulfur amino acid profile.

Alkaline extraction is a conventional processing method for sunflower protein industries, resulting in a 70 % higher protein yield than acidic extraction (Gonzalez-Perez & Vereijken, 2007). Chlorogenic acid (CGA), in sunflower seeds and meals, is often removed by de-phenolization or pre-treatment with organic solvent mixture or enzymes before alkaline extraction to prevent the oxidation that induces green discoloration (W. Zhang, Liu, Hu, & Yang, 2019). Consequently, the conventional pre-treatment of sunflower protein diminishes antioxidant properties due to the loss of phenolics (P. R. Salgado et al., 2012).

Currently, patented industrial sunflower protein extraction methods are based on organic solvent extraction (Claudia Pickardt, Eisner, Bader, Wild, & Muller, 2014), oxygen exclusion (Austrade), and acidic precipitation (Pearce, 1984; Petit, Davin, & Gueguen, 1979). Austrade's patented process for Heliator® sunflower protein flour (45% and 55% protein content) retains the polyphenols through cold pressing of sunflower seeds and oxygen exclusion. Therefore, a novel approach to achieve higher protein concentration while maintaining polyphenols is needed in the sunflower industry.

Liang and Were (2020) found that L- cysteine could completely inhibit greening by redox regeneration of CGA and the formation of thiol conjugates in buffered pH 8 and 9 CGA– lysine solutions. Similarly, Verena Bongartz et al. (2016) and (Schilling, Sigolotto, Carle, & Schieber, 2008) indicated that CGA-cysteine conjugates in sunflower meals were colorless, suggesting that using cysteine to trap highly reactive CGA quinones could inhibit greening during high pH sunflower protein extraction. Thiol groups of cysteine and glutathione (GSH) can nucleophilically conjugate with CGA quinones to inhibit TBA formation (Y. Liang & Were, 2020). Liang and Were (2020) also showed that CGA-cysteine conjugates enhanced the radical scavenging ability of CGA-Lys solutions with added cysteine after 48 hours. Therefore, incorporating thiol-containing antioxidant dough conditioners such as GSH or L-cysteine into alkaline sunflower protein extraction to prevent greening without de-phenolization by organic acid is proposed as a novel processing method.

This project's long-term goal is to determine the anti-greening and structural modification effects of GSH and L-cysteine in alkaline extracted sunflower protein. This overall goal was accomplished by determining the individual and combined effects of L-cysteine or GSH concentrations (0 to 5.6 mM) and pH (7, 9, and 11) followed by isoelectric precipitation on color, protein, and free CGA yield, structural modifications. Lower greening and concentration-dependent conformational protein changes were expected with increasing thiol concentrations during alkaline protein extraction.

Alkaline protein extraction has not been studied with non de-phenolized sunflower ingredients with the addition of thiol to de-green the protein, increase protein yield and evaluate structural modifications. The thiols (L-cysteine or GSH) in alkaline sunflower protein extraction could prevent phenolic oxidation by forming CGA-thiol conjugate. As thiol incorporation into

alkaline sunflower protein extraction will not require de-phenolization, this process should effectively result in sunflower protein with minimal greening and retain native protein conformation.

2. Literature Review

2.1. Composition of Sunflower Protein

Though sunflower cultivars contain different percent protein, the major sunflower proteins in sunflower seeds are helianthinin (40 – 90% of total seed protein) and albumin (SFA) at 10 – 30% of total seed protein (Gonzalez-Perez & Vereijken, 2007). Helianthinin and albumin differ in their molecular weight and pH-dependent solubility. Helianthinin is a globulin protein with a molecular weight of 300 to 350 KDa and is present in the 11S and 7S forms. In comparison, sunflower albumin (SFA) is a 2S lower molecular weight albumin protein of 10- 18 kDa (Gonzalez-Perez & Vereijken, 2007). Depending on the extraction method, sunflower protein might contain different helianthinin and albumin ratios, which could be identified by gel electrophoresis.

The isoelectric pH of helianthin is 4.0– 5.5 compared to the IEP of SFA, which is 8.8 (Gonzalez-Perez & Vereijken, 2007). The 11S helianthinin is extractable at neutral pH (6-7) and alkaline pH (8-11). However, at a higher pH (10-11), the 11S form of helianthin is likely to dissociate into trimeric 7S form (Figure 1), which can decrease protein functionality such as solubility and foaming (Molina, Petrucci, & Anon, 2004). At extreme pH (>12 and <3), dissociation of helianthinin into monomeric (3S) form occurs (Molina et al., 2004). Further acidification to a pH of 3.0 denatures helianthinin. Thus, extraction at a temperature of less than 65°C and alkaline pH ≥ 9 , followed by acidic precipitation at isoelectric pH (4.0 – 5.6), is recommended.

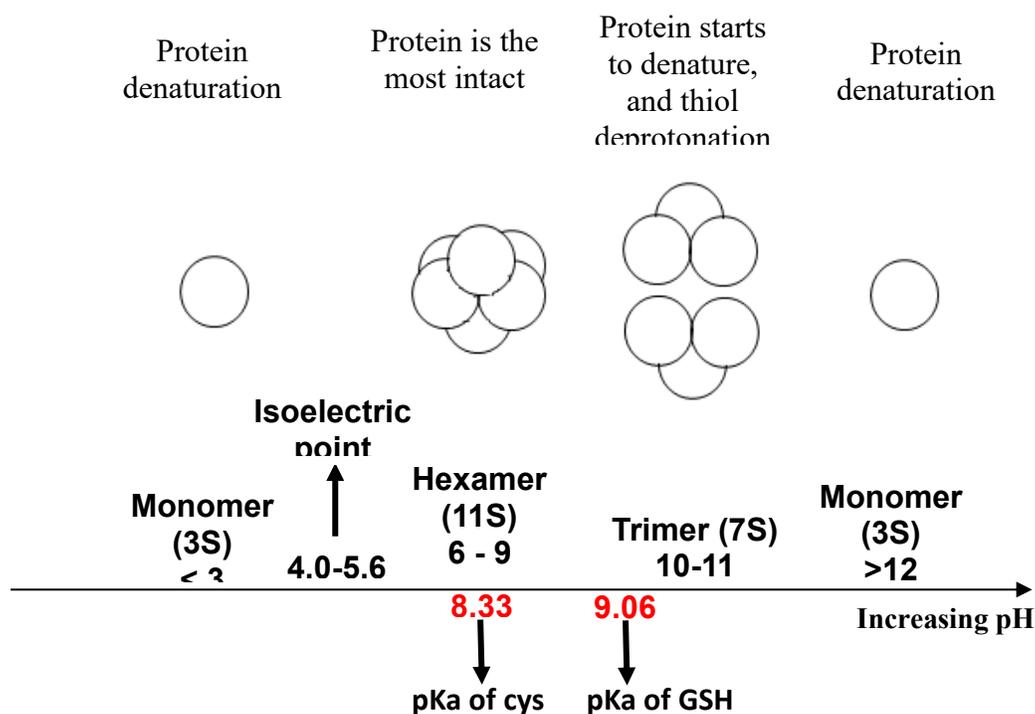


Figure 1 Helianthinin structure with increasing pH (Gonzalez-Perez & Vereijken, 2007; Molina et al., 2004) and predicted pK_a of cysteine and glutathione (Tummanapelli & Vasudevan, 2015)

In contrast with helianthinin, sunflower albumin (SFA) contains more sulfur-containing amino acids (16 methionine and eight cysteines, out of total 61 amino acids) as compared to helianthinin, which includes three methionine and six cysteines out of a total 481 amino acids (Pantoja-Uceda et al., 2004; Yonder Haar, Allen, Cohen, Nessler, & Thomas, 1988). However, since SFA only constitutes 10 – 30 % of total seed protein than 40-90% helianthinin, a lower yield is expected with SFA extraction. During extraction, SFA is soluble between pH 3 to 9 and high extraction temperature (>100 °C) compared to helianthinin, which denatures at pH 10 and temperature as low as 65°C (Gonzalez-Perez et al., 2004). Furthermore, acidic extraction can protonate amino acids in SFA isolate, which increases solubility over a broad pH range of 2 – 11 solutions due to the charged groupsz (Albe Slabi, Mathé, et al., 2020; Pérez, Vereijken, Koningsveld, Gruppen, & Voragen, 2005). Hence, in comparison with helianthinin, SFA isolate

has higher solubility, stability, and foaming due to less protein cross-linking (Gonzalez-Perez & Vereijken, 2007). Table 1 summarizes extraction methods for helianthinin and SFA.

Table 1 Extraction methods for helianthinin and albumin sunflower protein isolate

Extraction methods	Sunflower protein class	Advantages	Disadvantages	Citations
pH (7-9), followed by IEP at pH 4.0-5.6, Temperature < 65°C.	Helianthinin	Alkaline extraction favored helianthinin resulting in higher yield due to higher % helianthinin in sunflower seeds.	 Lower solubility than SFA (very insoluble at pH <5, highest solubility (>80%) at pH 7-9)  Helianthinin isolate overall contained a PDCAAS score of ≤ 0.6 than SFA isolate and other common plant proteins, and Less yield than Helianthinin as only 10-30% of total protein in sunflower meal is SFA	(Gonzalez-Perez & Vereijken, 2007; Molina et al., 2004).
Acidic pH of 4.1 to 6 and 0.25 M NaCl Low to high extraction temperature (25°C -100 °C)	Albumin	Isolate from SFA had high solubility at pH 2 to 9, higher emulsifying capacity, and foam stability than helianthinin		(Albe Slabi, Mathé, et al., 2020; Pérez et al., 2005)
Neutral to alkaline pH (7, 9, and 11) followed by acidic precipitation at IEP pH (5.6), and 25°C extractions.	Expected proteins to be extracted are helianthinin	Higher yield due to increased solubility by prevention of protein-phenol interaction by thiol addition.	pH 11 might lower solubility despite thiol addition due to denaturation of 11S form of helianthinin	Current study

2.2.Factors affecting sunflower protein extraction

Organic solvents, enzymatic hydrolysis, pH, salt, and temperature, the absence of oxygen affect protein extraction yield, color, and protein functionalities. In general, protein recovery during extraction can be lowered due to alkaline pH and concentrated solvent-induced denaturation. Thus, lowers final yield and protein functionalities, such as solubility and foam stability, are expected. Removal of phenolic acids (de-phenolization) from sunflower meal before protein extraction is most commonly accomplished using organic solvent mixtures (Table

3). This prevents not only oxidation that causes greening during alkaline pH, but also protein denaturation due to protein-phenolic interaction (Gonzalez-Perez et al., 2002).

2.2.1. Extraction pH and organic solvent mixture

Extraction pH is crucial in determining the yield and color of sunflower protein, as extracted helianthinin and albumin solubility are pH-dependent (Table 1). Plant proteins are traditionally extracted by solubilizing protein-rich ingredients at alkaline pH, followed by acidic isoelectric precipitation (Hadnađev Miroslav et al., 2017). However, higher pH extraction can cause oxidation of CGA, which will leads to green pigmentation and bitter taste in extracted protein (Hadnađev Miroslav et al., 2017; Xu & Diosady, 2002). Hence, it is important to prevent phenolic oxidation. In coffee processing, off-flavor due to phenolic oxidation is prevented by removing CGA by enzymatic hydrolysis before roasting (Kraehenbuehl et al., 2017). Similarly, CGA is removed by de-phenolization of sunflower seed meals before alkaline extraction (Gonzalez-Perez et al., 2002).

De-phenolization of CGA from sunflower meals is commonly accomplished using an organic solvent (60 – 80% methanol, ethanol, and 2-propanol) in aqueous solutions (Berot & Briffaud, 1983; Mudasir Ahmad Malik & Saini, 2017). These solvents differ in polarity and ionic strength (from most to least polar: methanol > ethanol > 2-propanol). Thus, different organic solvents will result in different percentages of removal of CGA. Low ionic strength (<250mM) solvent mixtures are preferable since protein denaturation and loss of albumin can be minimized (Gonzalez-Perez et al., 2002) as protein loss is likely as albumin has high solubility and could be co-extracted with high ionic strength solvents (Gonzalez-Perez et al., 2002).

Table 2 Selected studies for de-phenolization of sunflower meal using organic solvents

Organic solvent	% CGA removed	Additional benefits	Disadvantages	References
80% methanol	100%	No helianthinin denaturation	partial loss of 2S albumin, as confirmed by SDS-PAGE	(Gonzalez-Perez et al., 2002)
	94%	The presence of 30–40 kDa and 20–30 kDa bands indicated Sunflower albumin extraction in 2S form.	Protein concentrate exhibited low solubility in water (50%)	(P. R. Salgado, Molina Ortiz, Sara E., Petruccielli, Silvana, Mauri, Adriana N, 2012)
60% methanol	Total phenol At pH 5 and 7: 0.76 mg/mL pH9: 0.79 mg/mL (no initial CGA content displayed)	Similar protein content (data not shown) before and after de-phenolization of sunflower seeds	Low protein solubility (0.62 mg/mL in pH 7 water)	(De Leonardis, Macciola, & Di Domenico, 2005)
70% ethanol	93%	The presence of MW bands of 30–40 kDa and 20–30 kDa indicated extraction of 2S albumin	Protein concentrate exhibited low solubility in water (40%)	(P. R. Salgado, Molina Ortiz, Sara E., Petruccielli, Silvana, Mauri, Adriana N, 2012)
	pH 5: 97%	High protein solubility: 80% at pH 7, and protein digestibility: 95%		(Alexandrino, Ferrari, de Oliveira, de Cássia S.C. Ormenese, & Pacheco, 2017)
60% ethanol	Total phenol At pH 5 and 7:	Similar protein content (data not shown) before and after de-	Low protein solubility (1.09 mg/mL in pH 7 water)	(De Leonardis et al., 2005)

	1.05 – 1.09 mg/mL pH9: 1.20 mg/mL (no initial CGA content displayed)	phenolization of sunflower seeds		
60% acetone	Total phenol At pH 5 and 7: 0.76 mg/mL pH9: 0.79 mg/mL (no initial CGA content displayed)	Similar protein content (data not shown) before and after de-phenolization of sunflower seeds	Low protein solubility (1.20 mg/mL in pH 7 water)	(De Leonardis et al., 2005)
None applied as alkalized water was used	To be determined	Without organic solvents, protein denaturation and loss of yield and solubility could be minimized	Thiol might not entirely prevent phenolic oxidation, and phenolic-protein co-extraction is possible at higher pH.	This study

Gonzalez-Perez et al. (2002) achieved a 100% extraction of CGA from dehulled sunflower meal with 80% aqueous methanol with no protein denaturation. Also, De Leonardis, Macciola, & Di Domenico (2005) achieved minimal protein loss when 60% aqueous ethanol at pH 5 was used to remove CGA and caffeic acid from sunflower seed shells. Water can also remove CGA as CGA is soluble in water (Molina et al., 2004). However, water causes a high loss of water-soluble albumins, which can lower the stability of oil-water emulsion after pH 9 alkaline extraction (P. R. Salgado et al., 2012). In contrast, sodium sulfite used for alkaline extraction resulted in protein isolate with a more stable emulsion because disulfide bonds formation is retained through minimizing albumin loss (P. R. Salgado et al., 2012).

Different solvent mixtures used to de-phenolize sunflower meals, demonstrated in

Table 2, are needed for various extraction pH and processing purposes, such as extracting helianthinin isolate vs. albumin concentration. Nonetheless, alkaline pH generally results in a higher yield due to increasing helianthinin solubility at high pH (Section 2.1). In this study, Milli-Q ultrapure water was used for extraction, and 6 M NaOH was added to achieve specific extraction pH (7, 9, or 11). This method is hypothesized to extract both 7S or 11S forms of helianthinin, depending on thiol type, concentration, and extraction pH.

2.2.2. Enzymatic hydrolysis of CGA before alkaline extraction

Chlorogenic acid removal from sunflower seeds of 99% and 100% was achieved by W. Zhang et al., (2019) and Laguna et al. (2019), respectively, by using the CGA hydrolase enzyme (EC 3.1.1.42) prepared from *Aspergillus niger*. W. Zhang et al., (2019) found increased solubility, water absorption capacity, and emulsification stability of enzymatically hydrolyzed sunflower seed paste of 48%, 59%, and 23%, respectively, compared to control (un-dephenolized seed paste). Enzymatic hydrolysis can enhance functional properties such as solubility without protein loss or denaturation, which can be an alternative for de-phenolization with an organic solvent mixture (W. Zhang et al., 2019). In contrast with enzymatic hydrolysis, the current study added thiol which were hypothesized to increase protein solubility and soluble CGA through enhancing solubility by preventing protein-phenolic interaction.

2.2.3. Effect of extraction temperature on protein yield

Extraction temperatures of 65 and 90 °C could induce sunflower protein denaturation during extraction (Gonzalez-Perez et al., 2004). If high heat (80°) for a prolonged time (18h) is conducted, the alkaline extraction of SPI could produce lysinoalanine, which reduces protein digestibility and health concern as lysinoalanine causes renal tumor in rodents (Cheftel, Provansal, & Cuq, 1975; Chen et al., 2019). Thus, lower temperature and processing time might

increase protein extraction yield by preventing temperature-induced denaturation. Claudia Pickardt, Hager, Eisner, Carle, & Kammerer (2011) found that an extraction temperature of 8 °C resulted in 33% protein loss compared to 24 and 38°C, which resulted in > 40% protein loss.

After sunflower protein isolation, sunflower protein is dehydrated. Spray drying of sunflower protein flour lowers solubility and increases yield loss due to protein denaturation at 80 °C (Ermis & Karasu, 2020; Claudia Pickardt, Eisner, Kammerer, & Carle, 2015). In contrast, low-temperature lyophilization is more desirable in preventing protein denaturation and retaining the protein isolate's higher solubility. Hence, we lyophilized to prevent heat-induced denaturation in sunflower protein isolate.

2.2.4. Effect of salt during sunflower protein extraction on protein yield

Micellization, the use of salt for protein extraction followed by acidic precipitation, increases sunflower protein yield because helianthinin, the predominant sunflower protein (40 – 90%), is salt-soluble (Gonzalez-Perez et al., 2004). Incorporating 0.5 M NaCl during extraction could potentially increase protein yield due to extracting more helianthinin subunits. When Ivanova et al. (2012) investigated the effect of 0 – 20% NaCl at pH 2 to 10, they obtained 50% protein yield with a lighter color and diminished protein-phenol interaction using 10% NaCl, at pH 6 and 40 °C extraction. Gonzalez-Perez et al., (2004) achieved 60% protein yield at pH 10 and 7.5 to 12.5% NaCl concentrations. However, the protein extracts were dark brown due to protein-phenolic interaction.

When Albe Slabi et al. (2020) studied the effect of pH (3–6) and 0–0.5 M NaCl on sunflower albumin extraction, the highest extraction yield was 62% with pH 4.1 and 0.25 M of NaCl. Among the extracted protein, 88.78% were SFA with low protein-phenol interaction. Also, Claudia Pickardt et al. (2015) found that acidic (pH 6) extracted proteins with higher NaCl

concentrations of 1.3 and 2.0 M and polyphenol adsorption by resin for the SPI extraction and SFA concentrate. After sunflower press cake was immersed in 1.3 and 2.0 M saline solution, raffinination followed by adsorption resin to de-phenolize the protein extract was used. Next, the protein extract was precipitated by HCl to pH 3.5. The residue was purified and adjusted to pH 7 to recover protein isolate, while the supernatant was ultra- and dia- filtered to recover protein concentrate. The yield of SPI and SFA was 15% and 5%, respectively. Claudia Pickardt et al. (2015) suggested that lower protein isolate yield was due to acidic extraction favoring albumin extraction. \Claudia Pickardt et al. (2015) indicated that the sunflower press cake used in the study was exposed to high heat during industrial de-oiling, which denatures protein. Nonetheless, at pH 7, SPI's solubility and emulsifying capacity were 17.3 – 13% and 180 – 205 ml/g, while SFA's was 72.4 – 74 and 510-640 mL/g. Thus, acidic extraction and salt incorporation increase the SFA solubility while lowered SPI solubility due to helianthinin denaturation (Pérez et al., 2005).

Sunflower protein isolate extracted by Ivanova et al. (2012), Albe Slabi et al. (2020), Zhang et al. (2019), and Claudia Pickardt et al. (2015) all exhibited lighter color due to reduced protein-phenol interaction. Furthermore, SFA extracts from Albe Slabi et al. (2020) showed 100% solubility in water at a pH range of 2 – 11, while SFA concentrates from Claudia Pickardt et al. (2015) exhibited 74% solubility at pH 7. Regardless of salt's benefit during sunflower protein extraction, a protein extracted with high salt molarity will ultimately contain a higher sodium content, which could pose disadvantages in nutritional content from a consumer's perspective (Claudia Pickardt et al., 2015).

2.2.5. Thiol effect on protein extraction

In addition to organic solvents, enzymatic hydrolysis, incorporation of salt, extraction pH and temperature, the absence of oxygen (vacuum condition) during protein extraction can prevent discoloration from oxidized phenolic acids (Gonzalez-Perez et al., 2002). The use of thiol-containing antioxidants such as cysteine and glutathione could lower CGA oxidation when added before protein extraction due to lowered pH and their reducing capacity. Table 3 and 4 summarize acidic and alkaline conditions used for sunflower protein extraction.

Table 3 Selected studies of acidic sunflower protein extraction methods with corresponding protein yields

pH and NaCl concentration	(a) %de-phenolized or CGA content in protein and (b) color of protein	(a) %yield (from low to high pH) and (b)% SPI protein	(a) Advantages and (b) limitations	Citations
Acidic				
pH 5 + 0 M NaCl	a) Not reported. b) Not reported	a) Not reported. b) >95%	a) Sunflower albumin (SFA) had high solubility (>80%) between pH 3 to 9 and was not denatured with high heat (>100 °C) b) The SPI yield was considerably lower (data not shown) than helianthinin extract	(Pérez et al., 2005)
pH 3 to 6 + 0 to 0.5 M NaCl	a) 1.55 mg of CQA per g of SFA a) pH 4.1; 0.25 M NaCl= SFA: white (L* = 92.2 ± 2.9, a* = 1.3 ± 0.7, b* = 11.0 ± 1.4)	a) Helianthinin: 3% (pH 3) 20% (pH 6) Albumin: 50% (pH 3); 25% (pH 6) b) pH 4.1; 0.25 M NaCl= SFA: 93.3%	a. The yield of SFA increased with NaCl concentration. The most optimum SFA extraction condition based on response surface methodology analysis was pH 4.1 and 0.25 M NaCl b. The pH 4.1 and 0.25 M NaCl favors SFA extraction and causes low helianthinin extraction yield.	(Albe Slabi et al. (2020)
pH 6, followed by precipitation at pH 3 to 4.5 + 0 to 2.0 M NaCl	a) Not reported. b) Not reported	a. pH 3.5; 2.0 M NaCl= 23%; pH 4.5; 2.0 M NaCl= 19% b. pH 3.5; 2.0	a. Sunflower protein extract was light-colored and rich in albumin (data not shown) b. Low SPI yield due to partial denaturation in sunflower meal ingredient caused by the oil recovery process	(Claudia. Pickardt et al., 2011)

		M NaCl: 99%; pH 4.5; 2.0 M NaCl: 99%		
pH 6, followed by precipitation at pH 3 to 4.5 + 0.6 to 2.0 M NaCl	a) Not reported 99.4% reduction in CGA in SPI b) pH 3.5 & 0.6 M NaCl= white; (L*=82.8, a*=1.8, b*=12.7) ; pH 3.5 & 2.0 M NaCl= white (L*=86.8, a*=0.9, b*=10.2)	a) pH 3.5 & 0.6 M NaCl=11%; pH 3.5 & 2.0 M NaCl=23 % b) pH 3.5 & 0.6 M NaCl=96%; pH 3.5 & 2.0 M NaCl=99 %	a) SPI exhibited moderate emulsifying capacity (209-276 ml/g) and high foam stability (94-96%) b) The solubility of SPI was low (10.2-14.6% at pH 7). This low solubility was consistent with (Claudia. Pickardt et al., 2011)	(Claudia Pickardt et al., 2011)
pH 6 + 1.3 and 2.0 M NaCl, followed by centrifugation: a. The precipitate was isoelectric precipitated at pH 4.5 to extract protein isolate b. The supernatant was subjected to ultrafiltration and	a) SPI: <2% - not detected Albumin protein concentrate: <1% - not detected b) SPI: off-white to cream (L*=82.5-84.7, a*=1.6-1.7, b*=11.7-12.5); albumin concentrate = light grey to beige (L*=61.6-78.7, a*=1.4-2.0, b*=9.7-10.2);	a) SPI: 2.0 M NaCl = 23-26%;1.3 M NaCl =15%. Albumin protein concentrate: 5% b) SPI: 2.0 M NaCl = >94% ;1.3 M NaCl =15%. Albumin protein concentrate: 65-68%	a) Different kinds of concentrates (both SPI and albumin) can be extracted, and polyphenol from sunflower meal was adsorbed by resin for other industrial use. b) Despite the light color, SPI had very low solubility (7.3 – 13% at pH 7), while albumin concentrate had much higher solubility (72.4-74% at pH 7), which could be due to helianthinin denaturation from prolonged precipitation mixing time.	(Claudia Pickardt et al., 2015)

diafiltration to
extract protein
concentrate

- a) Not reported
- b) Light-colored

pH 6 + 0 M
NaCl

- a) protein
extract: 30%
- b) protein
extract:
35±4%

- a) This pilot plant study demonstrated the use of spray drying and acidic pH extraction for industrial production.
- b) Spray-dried sunflower extract exhibited low bulk flowability and low solubility due to heat-induced protein denaturation.

(Ermis &
Karasu,
2020)

Table 4 Selected studies of alkaline sunflower protein extraction methods with corresponding protein yields

pH and NaCl concentration	%de-phenolized or CGA content in protein (a) and color of protein (b)	(a) %yield (from low to high pH) and (b)%protein	Advantages (a) and limitations (b)	Citations
Alkaline				
pH 9 + 0 M NaCl	a) < 0.01% b) Light brown, creamy color	a) SPI: 15 ± 2 b) SPI: 98 ± 2	a) 100% of polyphenol was successfully removed by methanol, and alkaline extraction did not denature the protein isolate b) Protein extraction at pH 9 caused a partial loss of 2S albumin, as confirmed by SDS-PAGE	(Gonzalez-Perez et al., 2002)
pH 8.5 + 0 M NaCl	a) Not reported b) Not reported	a) Not reported b) Helianthinin: >85%	Denaturation temperature was 65 °C for 7S helianthinin and 90 °C for 11 S helianthinin, which both decreased the solubility of the protein isolate	(Gonzalez-Perez et al., 2004)
5 to 12+ 0.4 M NaCl	a) Not reported b) Not reported	Not reported.	a) Extreme alkaline pH (>11) denatured helianthinin b) There was no determination of yield from pH 5 to 12 + 0.4 M NaCl.	(Molina et al., 2004)
7, 8.5, and 10 + 0 M NaCl	a) Not reported b) pH 7 ->slight discoloration, pH 8.5 and 10 - > green pigmentation	a) Not reported b) Not reported	a) High pH caused CGA-protein binding, which decreased crude protein degradation and increased sunflower meal protein digestibility for ruminants by up to 10% b) CGA-protein-phenolic binding caused	(V. Bongartz et al., 2018)

	(observed at 680 nm wavelength absorbance)		greening, which is undesirable for human consumption	
6 to 9 + 0 to 0.5 M NaCl	<ul style="list-style-type: none"> a) < 0.02% b) White ($L^* = 82.3$, $a^* = 0.2$, and $b^* = 41.3$) 	<ul style="list-style-type: none"> a) Total protein: 45% b) Total protein content: 93% Helianthinin proportion: 57%, albumin: 43% 	<ul style="list-style-type: none"> a) A high proportion of albumin (41%) is retained within the SPI b) The SPI solubility was low (40%) on pH 5-7 due to the isoelectric point of helianthinin, while high on acidic and alkaline (80–100% at pH 2–4 and 8–11) 	(Albe Slabi, Mathe, et al., 2020)
10 + 10% NaCl solution	<ul style="list-style-type: none"> a) With succinic acid: CGA = 412.8 ± 20.31 mg/kg With Hcl: CGA = 590.8 ± 31.28 mg/kg b) Succinic acid: light (Hunter $L^* = 94.12 \pm 2.19$) Hcl: dark (Hunter $L^* = 53.15 \pm 1.26$) 	<ul style="list-style-type: none"> a) 46. and 50% b) 95% 	<ul style="list-style-type: none"> a) High SPI yield, and when 10% of SPI was used in bread making, the essential amino acid score increased by 13%. In contrast, valine, methionine, histidine, and arginine values increased by 16%, 20%, 29%, 33% (based on recommended daily value from Russian Federation). SPI processed with succinic acid de-phenolization resulted in intense acidity when used in bread formulation, which is beneficial for sourdough bread as fermentation time is decreased. b) There was no test conducted on protein functionalities of SPI. It is unclear whether this SPI could apply to other formulations like beverages, as this study focused on breadmaking use with 	(Shchekoldina & Aider, 2014)

		high acidity.	
Not reported (proprietary information)	Helioflar® 55: >51%	a) Sunflower protein flour made from cold-press meal preserved polyphenols through oxygen exclusion without the use of solvent b) Due to the presence of polyphenols, sunflower flour can produce greening under high pH baking applications	(Austrade)*
7, 9, and 11+ N/A	a) pH 7: 5.51% pH 7.59: 7.70% pH 9: 21.43% pH 11: 31.99% b) not conducted in this study	The use of thiols as TBA inhibitors does not require de-phenolization of sunflower meals, as CGA-thiol conjugates extracted protein concentrate or isolate will potentially be less green and brown.	This study

* based on Helioflor® technical specification by Austrade (2016)

2.3. Structural changes of sunflower protein from de-phenolization and protein-phenol bonding

Protein-phenolic interaction during sunflower protein extraction denatures protein and can result in loss of functional properties. Chlorogenic and caffeic acids are abundant, consisting of 70% of total phenolics in sunflower seeds (Weisz, Kammerer, & Carle, 2009). Protein secondary structural modifications caused by phenols or thiol conjugation can be investigated using spectroscopy methods. Functional properties of sunflower protein powders, such as solubility and surface hydrophobicity, decreased in sunflower protein isolates with phenolic compounds compared to sunflower protein isolates made from de-phenolized sunflower protein meals (M. A. Malik, Sharma, & Saini, 2016). Furthermore, protein solubility decreased with more protein-phenolic conjugation due to higher exposure of tryptophan residues, thus increasing hydrophobicity (D. Karefyllakis et al., 2018). Therefore, we hypothesized that the prevention of protein-phenolic conjugation with added thiol might increase protein solubility (Mejri et al., 2005).

Dimitris Karefyllakis, Altunkaya, Berton-Carabin, van der Goot, & Nikiforidis (2017) found structural modification on sunflower protein isolate was caused by protein-phenolic binding when de-phenolized sunflower protein was mixed with CGA in a 0.1 M pH 7 phosphate buffer solution and with added 0.1 M NaCl to promote non-covalent interaction. These protein-phenolic binding induced protein unfolding through a decrease in the α -helices and β -sheets in the secondary protein structure. When used as oil-water emulsion stabilizers, CGA-bound sunflower protein isolate had higher stability against coalescence than sunflower protein isolate made from de-phenolized sunflower meal due to more extensive CGA-protein complexation, which formed larger complexes and lowered free protein molecules. In addition to reduced

interfacial properties, water-binding capacity was higher for sunflower protein made from un-dephenolized meals than sunflower protein made from de-phenolized meals (Mudasir Ahmad Malik & Saini, 2017). Thus, protein-phenolic binding could enhance antioxidant delivery from SPI to the human digestive system, enhanced through the increased oil-water emulsion and water-binding capacity (Waraho, McClements, & Decker, 2011).

Regardless of the enhanced interfacial properties of protein-phenolic interactions, the drawbacks of darker colors, off-bitter taste, and diminished functional properties such as reduced solubility and foam stability could outweigh the potential benefits of high extraction yield. Thus, sunflower proteins' industrial processing is generally accomplished with the de-phenolization of sunflower meals (Gonzalez-Perez & Vereijken, 2007). The sunflower protein extraction using thiols proposed in this study could prevent protein unfolding due to phenolic conjugation, and more soluble CGA can be retained (Olesia et al., 2019).

2.4.Thiol inhibition of greening and protection of CGA antioxidants

The greening observed in sunflower protein isolated under alkaline pH or in many CGA-rich foods such as sweet potato and burdock when exposed to alkaline pH and oxygen during storage, baking or deep-frying is due to the formation of trihydroxy benzacridine (TBA) derivative from oxidized CGA – quinone reacting with primary amino acids such as lysine (Goro, Yukimichi, & Kazuko Namiki, 2001). Verena Bongartz et al., (2016); Goro et al., (2001); Y. Liang & Were, (2020) indicated the mechanism and pathways contributing to the TBA formation from CGA oxidation. At alkaline pH, CGA-quinone is more likely to be attacked by nucleophiles with free NH₂ (amine) groups, such as lysine, which are abundant in sunflower meals. As a result, greening can occur during the alkaline processing of SPI from non-dephenolized sunflower ingredients. Thus, de-phenolization by organic solvent or chlorogenic

acid hydrolase could prevent oxidation-induced greening of sunflower proteins. However, CGA antioxidants are lost during the de-phenolization process.

As an alternative to de-phenolization, greening could be prevented using acidic pH or acidic ingredients in SPI processing. Atonfack, Ataman, & Were (2019) inhibited TBA formation with added acidulants in cookie dough prepared with sunflower butter. However, as stated in section 2.2.1, alkaline extraction of SPI is beneficial due to higher protein yield from increasing helianthinin solubility. Thus, we investigated SPI alkaline processing using thiol-containing cysteine or glutathione.

Liang & Were (2020) did not detect greening when 2mM cysteine was added to the CGA: lysine solution. Since cysteinyl thiol from cysteine is more nucleophilic than lysyl ϵ -amines, the CGA-quinone is more susceptible to nucleophilic attack by thiol from cysteine or glutathione than amine group from lysine, resulting in colorless CGA-thiol conjugate and preventing the formation of green TBA (Figure 2)

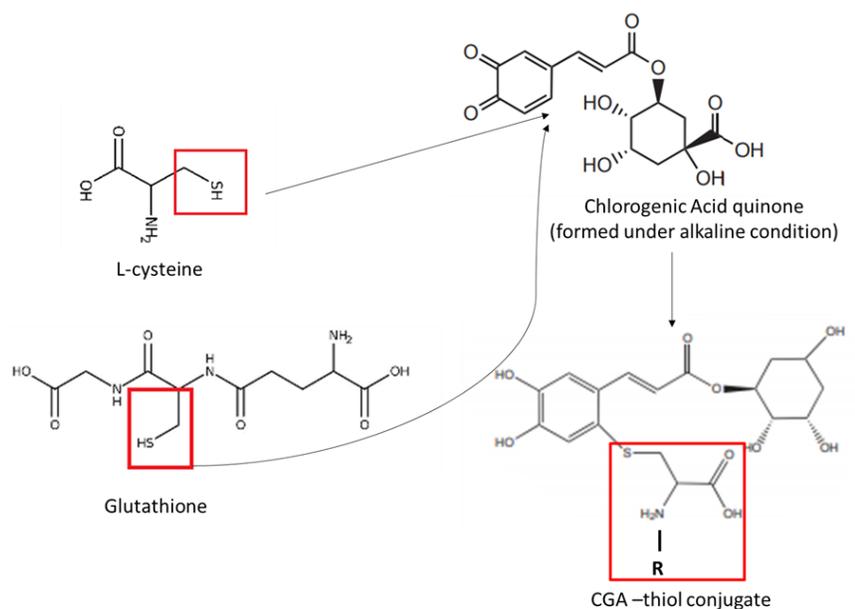


Figure 2 Mechanism of nucleophilic attack by cysteine or glutathione produced CGA – thiol conjugate (Y. Liang & Were, 2020). The red highlighted part indicates a thiol group.

Although L-cysteine is generally recognized as safe by the US FDA (21CFR184.1271), the use of cysteine in baking industries is controversial as 88% was manufactured from acid hydrolysis of keratin (animal byproduct), and 12% was manufactured from microbial fermentation in 2013 (Tamba Berehoiu, Popa, & Popescu, 2013).

In contrast to cysteine, glutathione (GSH), a tripeptide antioxidant composed of glutamate, cysteine, and glycine, is available from animal tissues (beef and fish muscles and liver) and plants, such as asparagus and avocado, and commercially manufactured from the fermentation of brewer's yeast (H. J. Forman, H. Zhang, & A. Rinna, 2009). As an antioxidant, glutathione can protect caffeic acid and its esters against non-enzymatic oxidation in wine, which lowers the sulfur dioxide that causes many wine consumption-induced sensitivities (Kritzinger, Bauer, & Du Toit, 2013; Vally & Misso, 2012). The GSH and cysteine could be applied in alkaline sunflower protein isolate to inhibit TBA formation. Therefore, with thiol addition during alkaline processing, greening inhibition could be achieved in sunflower protein isolate without de-phenolization of sunflower meal (Y. Liang & Were, 2020), while maintaining a high protein yield and increasing antioxidant from CGA-thiol conjugate.

The use of thiols in alkaline sunflower protein isolation to inhibit greening without de-phenolization has not been investigated. Cysteine is sourced from animal keratin, and thus, bakery industries have been replacing cysteine with yeast-derived glutathione as a dough conditioner to address vegan-friendly label demand (Gelski, 2018; Sherred, 2019). Glutathione, which is sourced from yeast extract's microbial fermentation (Liu, Zhu, Lian, Huang, & Xu, 2019), is more clean-label friendly than cysteine. Glutathione has been used as a dough conditioner in bakery industries and a preservative in the wine industry (Kritzinger et al., 2013).

The incorporation of GSH in alkaline sunflower protein isolation could be a clean label de-greening alternative to cysteine (Table 5).

Table 5 Benefits of alkaline processing of sunflower protein isolate with added thiols

Benefit	Alkaline processing of sunflower protein from non-dephenolized sunflower meal with added thiols
Technical	<ul style="list-style-type: none"> <li data-bbox="431 520 1365 625">■ Thiol inhibition of greening in sunflower protein isolate due to TBA inhibition (Y. Liang & Were, 2020) could replace the de-phenolization of sunflower meal by using organic solvent. <li data-bbox="431 627 1365 730">■ Enhanced protein yield through increasing solubility by preventing protein-phenol interaction through thiol addition (D. Karefyllakis et al., 2018).
Economical	<ul style="list-style-type: none"> <li data-bbox="431 741 1170 768">■ Cysteine is cheaper than glutathione (Scientific, 2021) <li data-bbox="431 770 1330 951">■ Sunflower meal is cheaper than other soybean meal (average of \$278/ton of sunflower meal vs. \$437/ton of soybean meal as of January 2021 (USDA, 2021). Thus, if greening can be inhibited by thiols, sunflower protein can be a more economical plant-based protein option.
Consumer perception	<ul style="list-style-type: none"> <li data-bbox="431 961 1354 1026">■ Sunflower protein is non-GMO, as no GMO sunflower are currently available (Ivanova et al., 2012). <li data-bbox="431 1029 1354 1134">■ Unlike soy, sunflower protein is not a major food allergen in the United States (FDA, 2004), making it an attractive protein source if the greening can be controlled. <li data-bbox="431 1136 1354 1241">■ Cysteine is generally recognized as safe (GRAS) by the US FDA for dough, strengthening at a usage limit of 90 ppm in bakery products (FDA, 2019). <li data-bbox="431 1243 1354 1356">■ Glutathione is a more vegan-friendly clean-label ingredient, as it is commercially manufactured from yeast fermentation, while most cysteine is manufactured from animal origin (Olesia et al., 2019).

2.5. Sunflower protein use in industry and application challenges

As the third-largest oilseed crop, the world production of sunflower oilseeds products (sunflower oilseeds, meal, and oil) increased by 10% between 2015 – 2020 (USDA, 2021).

Globally, the sunflower seeds market for human consumption is expected to reach \$30.11 billion by 2025, and a 3.4% annual growth rate is estimated from 2017 to 2025 (Hexa-Research, 2019).

Neutral pH extraction with 7.5 -12.5% NaCl exhibited high sunflower protein yield (>50%), and the extracts displayed high solubility and water-holding capacities, which could be applied to beverage formulation and function as a thickening agent (Ivanova et al., 2012). Dehulled sunflower seeds contain 20.4 – 40% protein by weight on a dry basis (Gonzalez-Perez & Vereijken, 2007), and companies like Austrade have patented technology for the industrial production of sunflower protein from defatted sunflower seeds (Doris, 2017). Planetarians worked with Barilla to manufacture high-protein green to dark dough products using sunflower protein (Katy, 2019). While upcycled sunflower products have nutrient, economic, and sustainability advantages, the oxidation-induced greening when used in high pH formulations, such as during the baking of cookies (S. Liang & Were, 2018), muffins (Grasso, Liu, & Methven, 2020), or biscuits (Grasso, Omoarukhe, Wen, Papoutsis, & Methven, 2019) could limit its' use in the food industry. The TBA does not pose any significant toxicity concerns. Iacomino et al., (2017) used a cytotoxicity assay when cells 1% CGA with bovine serum albumin was incorporated into HepG2 human intestinal, and the results confirmed that there was no cytotoxicity. However, the green color induced from TBA formations can be a drawback for some food applications.

2.6.Rationale and significance

This thesis supports the 2021 NIFA Priority Code - A1364 - Novel Foods and Innovative Manufacturing Technologies by developing innovative manufacturing technologies of alkaline extraction of sunflower protein isolate with the addition of thiol that inhibits greening of sunflower protein isolate while improving production efficiency and yield by replacing organic solvent de-phenolization process, thus preventing more protein loss (USDA, 2020a; W. Zhang et al., 2019). This novel approach allows for the formation of CGA-thiol conjugate, which inhibits

green TBA formation (Y. Liang & Were, 2020) while preventing protein-phenolic interaction and enhancing protein yield through increasing solubility (Albe Slabi, Mathé, et al., 2020; P. R. Salgado et al., 2012)

The overall research goal was to investigate how different thiols (L-cysteine and glutathione) and concentrations react with phenolic compounds during alkaline sunflower protein extraction. We accomplished this overall goal through the following aims:

- I. Demonstrate if adding thiols increases soluble protein and CGA. It was hypothesized that adding thiols during sunflower protein extraction would increase soluble CGA content by preventing TBA formation (Y. Liang & Were, 2020) and increases solubility by preventing CGA-protein conjugates (D. Karefyllakis et al., 2018).
- II. Determine the effect of extraction pH and thiol concentration on both greening and Maillard browning of sunflower protein powders. We hypothesized that a higher thiol content would decrease greening through the formation of colorless CGA-thiol conjugates (Y. Liang & Were, 2020). Notwithstanding increased browning might occur as amine groups in cysteine and GSH might participate in Maillard browning (Lund et al., 2017).
- III. Investigate changes in protein secondary structure as a function of cysteine and GSH concentrations and extraction pH. We hypothesized that higher cysteine concentrations (4.8 and 5.6 mM) and higher alkaline pH (>10) might unfold proteins due to increasing thiol reactivity (Nguyen, Streicher, & Anema, 2018), while GSH might hinder the degree of unfolding at alkaline pH (Henry Jay Forman, Hongqiao Zhang, & Alessandra Rinna, 2009)

Alkaline extraction of sunflower protein with the addition of thiol-containing bakery ingredients was expected to inhibit the greening of extracted proteins. *With the prevention of greening, sunflower protein powders can be used in a broader range of formulations to achieve organic and allergen-free status.* Thiols addition can increase the marketplace's availability and acceptability of sunflower proteins with less effluent waste than the use of organic solvent for de-phenolization in applications where greening was unwanted.

3. Materials and Methods

3.1. Materials

Organic sunflower flour (Heliaflor[®] 55) manufactured by excluding oxygen with CO₂ extraction to obtain a protein content of 55% was purchased from Austrade Inc. (Palm Beach Gardens, Fla., USA.). We purchased a PageRuler™ Plus prestained protein ladder (10 to 250 kDa), chlorogenic acid (98%), HPLC grade acetonitrile, and HPLC water from Thermo Fisher Scientific (Hampton, NH). A Luna Omega[®] 5 μ C18 (2) 100 Å (150 × 2 mm, 1.5 μ m particle size) column was purchased from Phenomenex[®] (Torrance, CA), L-cysteine hydrochloride of 98% purity instead of L-cysteine was used due to its high solubility. Thiols of 98% purity were purchased from Acros Organics (New Jersey, USA).

3.2. Experimental design and sunflower isolate preparation

Alkaline extracted sunflower isolate was prepared with Heliaflor[®] sunflower protein flour and 0– 5.6 mM L-cysteine HCl and reduced GSH were added (Figure 3). Heliaflor[®] 55: de-ionized water A (1:10) similar to ratios used by Albe Slabi et al. (2020), Claudia Pickardt et al. (2011), and Weisz, Carle, & Kammerer (2013).

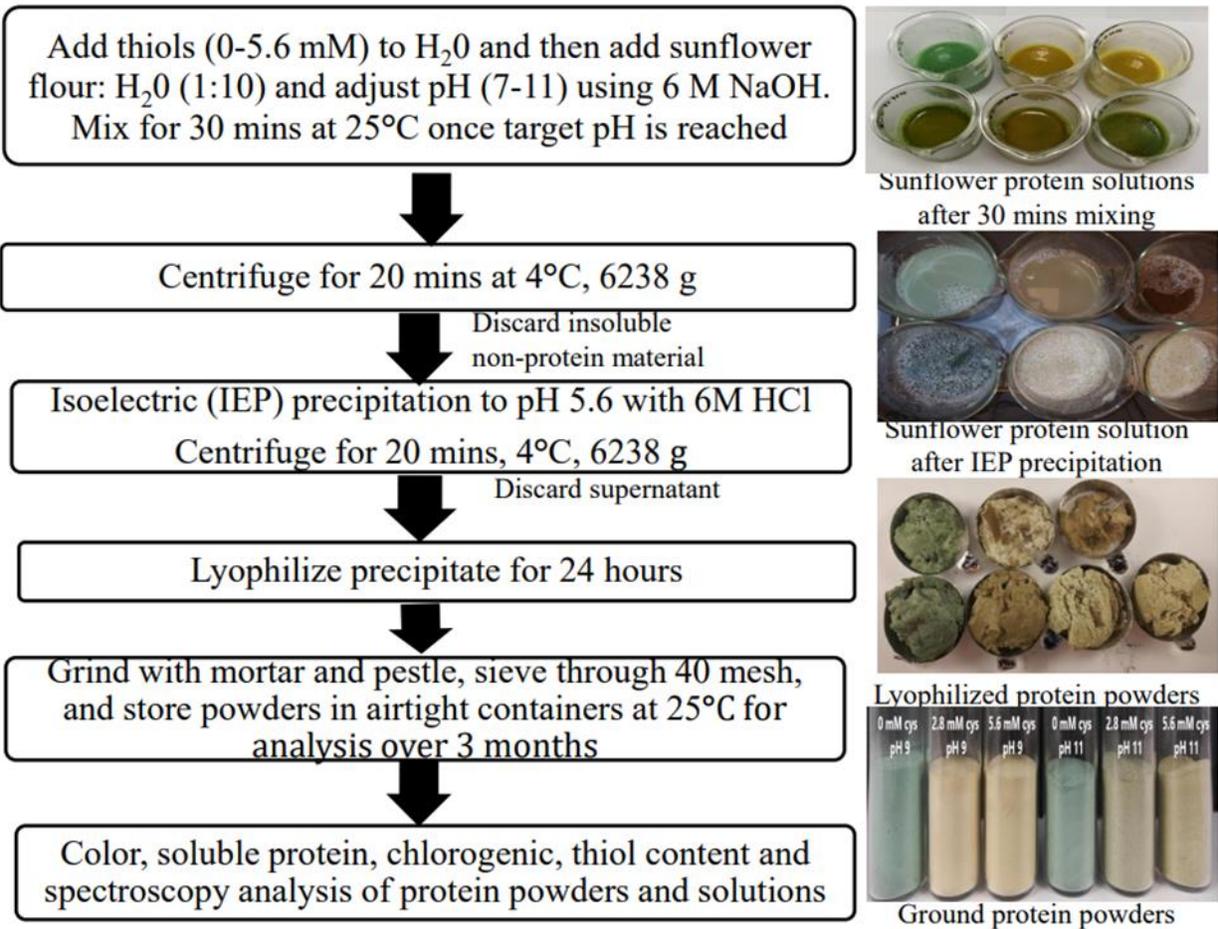


Figure 3 Alkaline sunflower protein extraction procedure to determine the effect of thiols

Thiol solutions (cysteine-HCl or GSH) were first added to Milli-Q water. Four sets of 450 ml thiol solution were placed in a 1-liter glass beaker on individual Cimarec+™ Stirring Hotplates Series (Thermo Fisher Scientific, Hampton, NH), and stirred at 850 rpm. In each glass beaker, 50 g of Heliator® 55 was then added with constant stirring. The mixing was performed at 25°C, and mixtures were adjusted with 6M NaOH to pH ranges from 7 to 11, as randomly assigned with RSM. Once the pH stabilized, the solution was mixed for 30 mins, and pH was checked and readjusted every 10 mins or as needed.

The mixtures were centrifuged at 6238 g at 4°C for 20 mins using an X1R centrifuge (Thermo Scientific, Germany). The supernatants with solubilized proteins were isoelectric precipitated to pH 5.6 by adding 6M HCl, followed by centrifugation at 6238 g at 4°C for 20 minutes to extract helianthinin, and some minor fractions of albumin as described by Gonzalez-Perez et al., (2004).

The precipitate was lyophilized for 24-30 h using the food profile:500 min ramp rate, -30°C freezing temperature for 9 h, followed by gradually increasing heating and sublimation temperature to 20°C, 40°C, 60°C, 90°C, 120°C, and holding for 15 hours (Harvest Right, Utah, USA). The mass of dried sunflower proteins was recorded, and the dried precipitate was ground with a mortar and pestle and sifted through a #40 mesh (0.841 mm) sieve. Protein solubility and structure modification induced by adding L-cysteine or GSH were then evaluated. Additionally, 0.1 g of protein powders were solubilized in 0.1 M pH 7 phosphate buffer to quantify soluble CGA by HPLC, and thiol and protein concentrations by DTNB thiol oxidation and Bradford assay, respectively (Figure 3)

3.3.Greening and browning color measurement

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 sunflower protein flour and isolate. The spectrophotometer has an aperture of 8 mm, an observation angle set at 10°, a D_{65} illuminant and white and black plates were used to calibrate the spectrophotometer (S. Liang & Were, 2018). Browning index was calculated from Hunter L*a*b* (Maskan, 2001) by using equation: $BI=100 \times ((X-0.31)/0.17)$, where $X= (a^*+1.75L)/[(5.645L+a^*-3.013b^*)]$

3.4. Protein and free thiol concentration of sunflower protein powders

Sunflower protein powder (0.1 g) was solubilized in 0.1 M phosphate buffer, pH 7, and protein concentration was measured using the Bradford method (Bradford, 1976). Thiol concentrations were quantified using DTNB thiol oxidation assay (Hawkins, Morgan, & Davies, 2009). TECAN Spark[®] (Tecan Trading AG, Switzerland) microplate reader was used in both assays.

1976). Coomassie blue stain dye (BioRad, United States) was diluted with DI water 4:1. 0 to 1.6 mg/ml bovine serum albumin (Thermo Scientific, Germany) was used to make the standard curve. The solubilized sunflower protein solution was diluted 1:2 with 0.1 M Phosphate buffer, pH 7 due to high absorbance value. 20 μ L of BSA and sunflower protein solution were each stained with 1 ml of 1x concentration of coomassie blue and incubated for 5 mins at room temperature. 200 μ L of stained samples were used for absorbance reading at 595 nm.

Cysteine hydrochloride or reduced glutathione from 0 to 0.4 mM were used as a standard curve, depending on the type of thiols used in the powder extraction. Ellman's reagent / 5,5'-dithiobis 2-nitrobenzoic acid (DTNB; Sigma Aldrich, Germany) solubilized in 0.1 M pH 7.4 phosphate buffer, was used to quantify protein thiols. Each solubilized protein sample was diluted with Milli-Q water to 1:2 and 1:4 ratio for the addition of DTNB and 0.1 M pH 7.4 phosphate buffer, respectively. 20 μ L of the diluted samples and 200 μ L of DTNB or 0.1 M pH 7.4 phosphate buffer were added in the microplate wells, followed by incubation in the dark in a microplate shaker for 30 mins at room temperature. Thiols were quantified from subtracted absorbance values of DTNB samples and phosphate buffer samples at 412 nm. All thiol concentrations were expressed as μ mol/mg of protein.

3.5. Quantification of Unreacted CGA by HPLC

Sunflower protein powders (0.1 g) were each dissolved with 10 ml 0.1 M phosphate buffer (pH 7), following method from Albe Slabi, Mathé, et al., (2020) and vortexed for 10 sec before filtering through a 0.2 μm syringe filter (Thermo Fisher Scientific, Hampton, NH). All samples were further diluted (samples: HPLC-grade water 1:4) before HPLC analysis (Figure 4).

Chlorogenic acid (CGA) was quantified as outlined by Y. Liang & Were, (2020) using an external standard curve of 0 to 0.1 mM CGA solutions diluted from 0.1 mM CGA solution (0.0354 g CGA in 10 ml HPLC water).

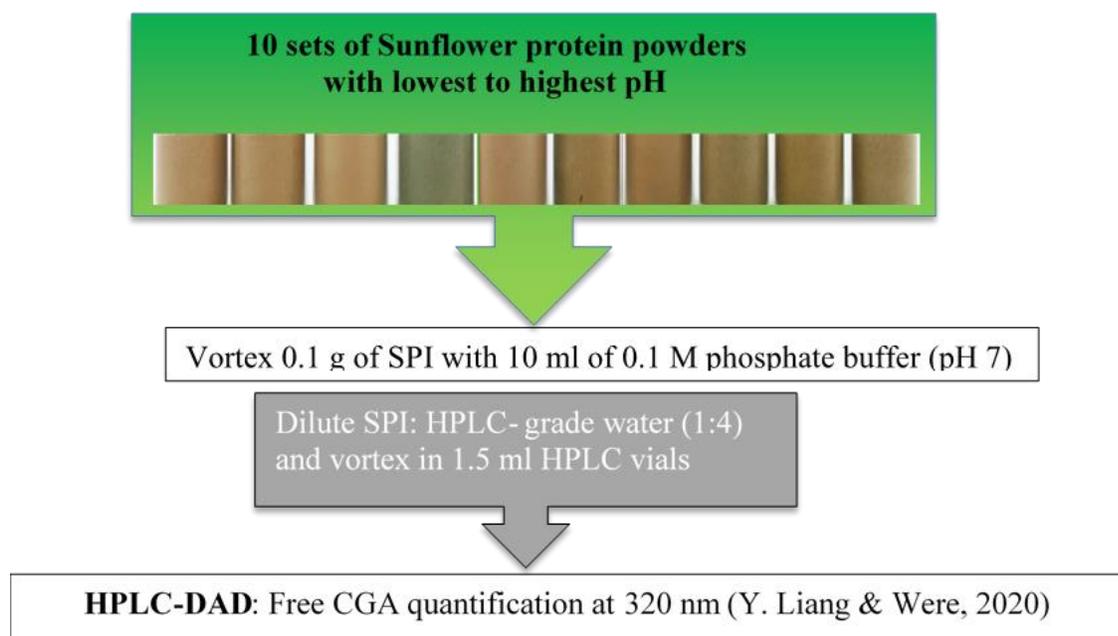


Figure 4 Quantification and identification of free chlorogenic acid using HPLC

Free soluble CGA was quantified using Agilent 1100 series HPLC instrument (Santa Clara, CA, USA) with a G1315B diode array detector. A Phenomenex® Luna Omega 5 μ C18 (2) 100 Å (150 \times 2 mm, 1.5 μm particle size) column was set at 30 °C. Wavelengths of 320 nm and a flow rate of 0.3 mL/min were used. HPLC-grade water with 0.1% formic acid and HPLC-grade acetonitrile with 0.1% formic acid were used for mobile phases A and B, respectively. The

gradient was 0 min, 0% B; 1-1.5 mins, linear gradient from 0% to 15% B; 1.5 - 4.5 mins, linear gradient from 15% to 30%; 4.5 to 6.0 mins 30% to 45% B; 6 - 8 mins, linear gradient 45% to 100% B; 8 - 9 mins isocratic on 100% and 9 -10 mins 0%.

3.6. Identification of helianthinin and albumin by SDS-PAGE

SDS-PAGE of sunflower protein solutions was performed according to Albe Slabi, Mathé, et al., (2020), except 12% instead of 14% polyacrylamide gels were used due to sharper gel quality. 0.1 mol/L of SPI was dissolved in deionized water. The sample was then solubilized in 50 μ L of Laemmli buffer containing 2% β -mercaptoethanol (v/v) and heated at 95 °C for 5 min. Molecular weight markers ranging from 10–250 kDa (PageRuler™ Plus Prestained Protein Ladder) were used (Bio-Rad, Hercules, USA). 25 μ L of aliquots for each sample were loaded onto a 4% polyacrylamide stacking gel/12% resolving gel. The gel was run at 200V for 75 min. A solution of 0.1% coomassie blue, 40% methanol, 10% acetic acid was used to stain the gel, followed by de-staining in a solution of 40% methanol and 10% acetic acid. The gel imaging was performed with the ChemiDoc XRS+ system with Image Lab software (S. No. 721BR09618, Universal Hood II, USA) to detect helianthinin (20 -44 KDa) and albumin (10-15 KDa).

3.7. Modifications in protein structure by protein-phenolic-thiol interactions

IRAffinity-1S Fourier-transform infrared spectrophotometer from Shimadzu (Shimadzu, Kyoto, Japan) with the ATR accessory was used to monitor secondary structure changes induced by extraction pH and thiol addition (Mudasir Ahmad Malik & Saini, 2018). 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 performed for each sample scan to prevent interferences from previous samples. The region in focus was amide I (1600 – 1700 cm^{-1}), amide II (1500 – 1600 cm^{-1}), and 1410 – 1310 cm^{-1} ,

as changes in absorbance as these regions correlate with protein-phenol conformational changes (D. Karefyllakis et al., 2018; Mudasir Ahmad Malik & Saini, 2018; Zhao, Mu, Zhang, & Richel, 2019).

To complement FTIR analysis, a Jasco J-1500 circular dichroism (CD) spectrometer (Jasco Co. Ltd., Tokyo, Japan) was also used to evaluate secondary structure modifications. Powders were diluted to 1mg/mL with deionized water, vortexed for 10 sec, and transferred into a quartz cuvette. Duplicate measurement of molecular ellipticity in far-UV (170–260 nm) and near-UV (260–350 nm) regions with a scan rate of 100 nm/min were performed based on average of five scans.

A FluoroMax-4 Spectrofluorometer (Horiba Scientific, Edison, NJ, Japan) was used to observe tertiary structure changes in protein solutions prepared in the same dilution as for CD analysis. Changes in fluorescence intensity as a function of increasing thiol concentration were monitored using an emission slit set at 2 nm, and fluorescence emission spectrum measured from 300 to 540 nm using excitation at 295 nm as described by Karefyllakis et al. (2018).

3.8. Statistical analysis

Response surface methodology (RSM) was used to evaluate the effect of two factors: pH (7.0 to 11.0) and thiol (cysteine and glutathione) concentration (0 to 5.6 mM). R statistical software (R Core Team, 2020) was used to randomly assign ten pH (7.0 – 11.0) and thiol concentration (0 – 5.6 mM) combinations and each set was performed in duplicate. Contours plots were made from observed greening and browning values, and results are considered significant if $P < 0.05$. The models were:

$$\text{a) greening} \sim b_0 + b_1 * \text{pH} + b_2 * \text{Concentration} + b_{22} * \text{Concentration}^2 + b_{12} * \text{pH} * \text{Concentration}$$

and

$$\text{b) } \text{browning} \approx \text{b}_0 + \text{b}_1 * \text{pH} + \text{b}_2 * \text{Concentration} + \text{b}_{22} * \text{pH}^2 + \text{b}_{22} * \text{Concentration}^2 + \text{b}_{12} * \text{pH} * \text{Concentration}.$$

Differences in hunter a* and browning index among treatment groups were assessed monthly for three months using the Kruskal Wallis test ($P < 0.05$).

4. Results and Discussion Soluble CGA and thiol quantification from solubilized powders extracted at pH 9

The water activity in lyophilized powders of 0.323 ± 0.080 was not significantly different among treatments. Hence, a_w was dismissed as a confounding factor affecting colors. The protein yield increased with pH and ranged from 5.51% (pH 7) to 31.99% (pH 11). The addition of thiol did not change the protein yield. Due to a complete set of concentrations (0 to 5.6 mM) from the RSM, we used pH 9 powders to evaluate the effects of thiols on protein and soluble CGA. The free soluble CGA at 320nm determined using HPLC showed that the highest soluble CGA was in powders with 2.8 mM Cys and GSH and not 5.6 mM thiols, which could be due to thiols acting as reducing agents at 2.8mM. In contrast, more CGA and thiol conjugation may have occurred with 5.6 mM thiols. However, this CGA-thiol conjugation did not result in different greening between 2.8 and 5.6 mM powders (Section 4.2; Table 7 and 8; D. Karefyllakis et al., 2018).

Higher thiols were detected in samples with more added thiols as expected (Figure 5). There was, however, no significant increase in % protein with added thiols during extraction. This lack of solubility increase was in contrast to Mejri et al., (2005), who reported that adding cysteine increased hydrolyzed gluten's solubility at pH 8-10 due to reducing disulfide bonds.

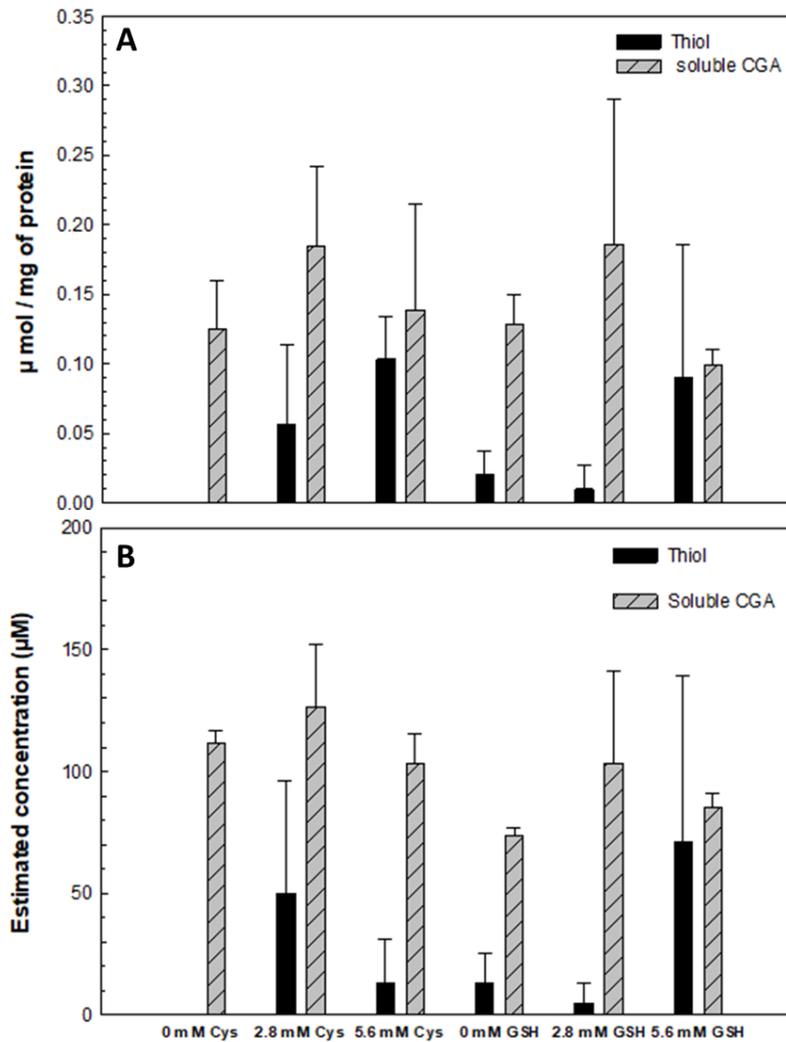


Figure 5 Thiol and soluble CGA of sunflower powders extracted at a target pH of 9 with 0, 2.8, and 5.6 mM Cys or GSH solubilized in 0.1 M pH 7 phosphate buffer; adjusted by protein concentration (A) and unadjusted (B).

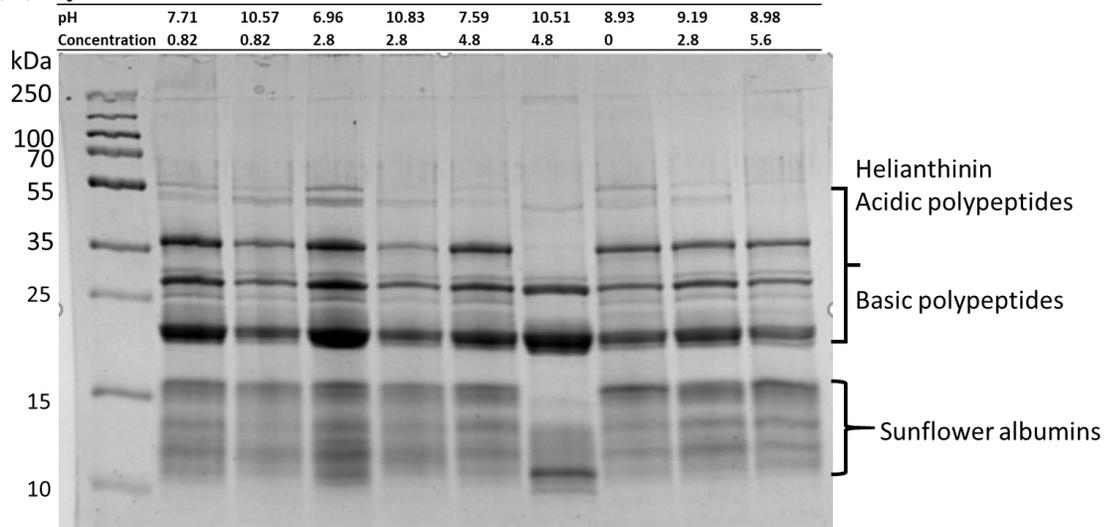
4.2.SDS-PAGE

Albumin (10-15KDa) and helianthinin with acidic (30-44 KDa) and basic (20-27 KDa) polypeptides linked by a disulfide bond, were present in all pH 7-11 samples. As expected, SDS-PAGE confirmed that more helianthinin than albumin proteins were extracted regardless of pH. Higher pH decreased the intensity of acidic helianthinin bands (30-44 KDa; lanes 3, 5, and 7) compared to lower pH (6.96-7.71; lanes 2, 4, and 6), which was possibly related to alkaline pH-

induced dissociation from 11S (contain both acidic and basic polypeptide) to 7S helianthinin form, which only contains basic polypeptide (Gonzalez-Perez & Vereijken, 2007).

At pH 7 to 9, there was no observable difference in intensity of 11 S helianthinin bands (20 – 44 kDa) with increasing cysteine (lanes 2, 4, and 6 and lanes 8, 9, 10). Nonetheless, at pH 11, the highest cysteine (4.8 mM) resulted in a shift from 11S to 7S helianthinin form, demonstrated by a lack of acidic polypeptide bands (30-44 KDa; lane 2, 3, and 5). The opposing results of cysteine and GSH is our recommendation for future research focus. This was in contrast with the highest GSH at pH 11, which resulted in the thickest acidic polypeptide bands (30-44 KDa), which could be due to oxidation of glutathione (GSSG) at higher alkaline pH, which favors disulfide bonds (Christopher, Anthony, & Harvey, 1992). Yano, (2012) found that reduced glutathione (GSH) was much more potent than oxidized glutathione (GSSG) in cleaving disulfide bonds in solubilized rice batter due to reduced activity. Hence, at alkaline pH (>10,41), glutathione was a better thiol to retain the native 11S helianthinin (20-44 KDa).

(A) Cysteine



(B) Glutathione

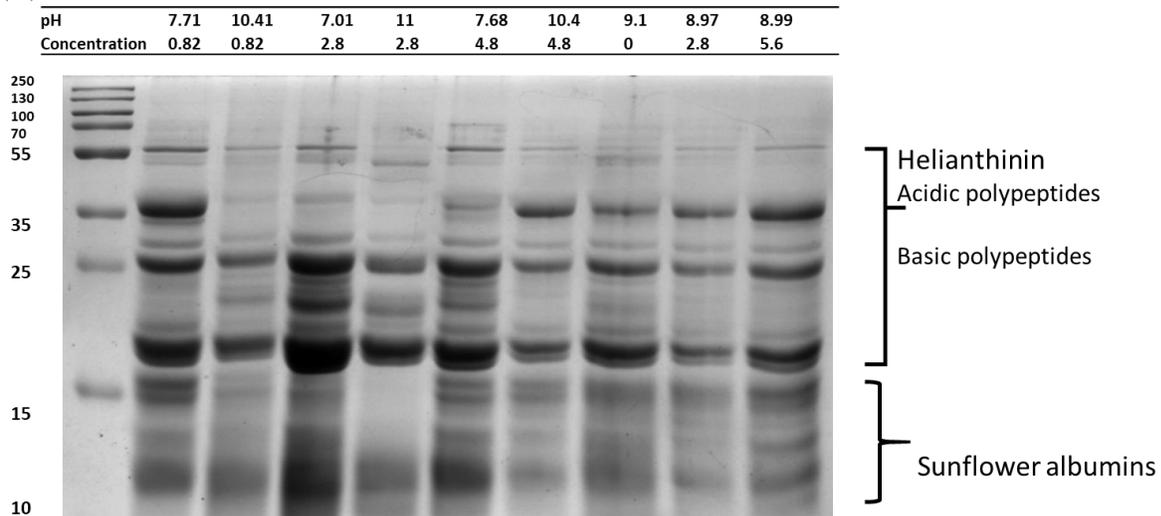


Figure 6 SDS-PAGE of sunflower protein isolate prepared with varying pH (7.58 to 11) and thiol concentration (0 to 5.6 mM) of (A) Cysteine and (B) Glutathione

4.3. Greening intensity as a function of pH, thiol type, and concentration

4.3.1. Effect of pH

Our Response Surface Methodology (RSM) design was randomized with varying pH (7 to 11) and thiol concentration (0 to 5.6 mM) to model greening inhibition, represented by higher Hunter a*. We failed to reject the lack of fit test ($p \geq 0.05$) from the contour plots of greening and

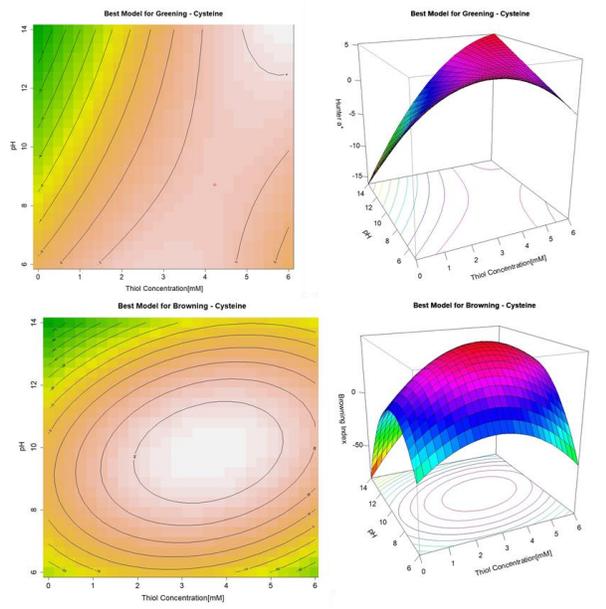
browning, thus confirming that the relationships assumed in the model were appropriate for predicting greening inhibition and browning for both cysteine and glutathione (Table 6).

Table 6 Regression coefficients of the predicted polynomial models for the color of sunflower protein based on thiol type, pH, and thiol concentration

	Hunter a*		Browning Index	
	Cysteine	GSH	Cysteine	GSH
R ²	0.7263	0.8722	0.5804	0.8114
Lack of Fit (p*)	0.7367	0.668	0.5287	0.3421
Intercept	9.499	26.921	-293.365	-13.397
pH	-1.83	-4.339	66.32	11.975
pH ²	NA	NA	-3.659	-1.2564
Concentration	0.5688	-4.175	8.9544	-17.5089
Concentration ²	-0.5132	-0.7409	-3.4364	-1.8098
pH*Concentration	0.4328	1.14842	1.5995	3.61586

Our model suggested that the greening decreased with extraction pH at higher cysteine or GSH concentration (Figure 7). The pH coefficient was more negative (-4.339) with GSH than cysteine (-1.83), and the coefficient of determination (R²) for Hunter a* in the RSM model for glutathione was higher (R²=0.8722) than cysteine (R²=0.7263) (Table 6). Thus, powders with GSH green more with increasing pH. The most optimum conditions indicated by the RSM model for the highest greening inhibition were pH 8.71 and 4.23 mM for cysteine and pH 8.51 and 3.78 mM GSH, which is also close to the pKa of Cys (8.18) and GSH (pH 9.06) (Tummanapelli & Vasudevan, 2015). Therefore, our model supports the hypothesis that adding thiols during alkaline protein extraction significantly inhibited greening (Y. Liang & Were, 2020). However, the contours plot displayed distinct differences in browning as a function of alkaline pH. With cysteine, browning peaked at pH 9.84 and 3.59 mM, whereas with GSH, browning peaked at pH 5.02 and 0.18 mM. Thus, alkaline conditions caused browning with cysteine but not with GSH.

(A) Cysteine



(B) Glutathione

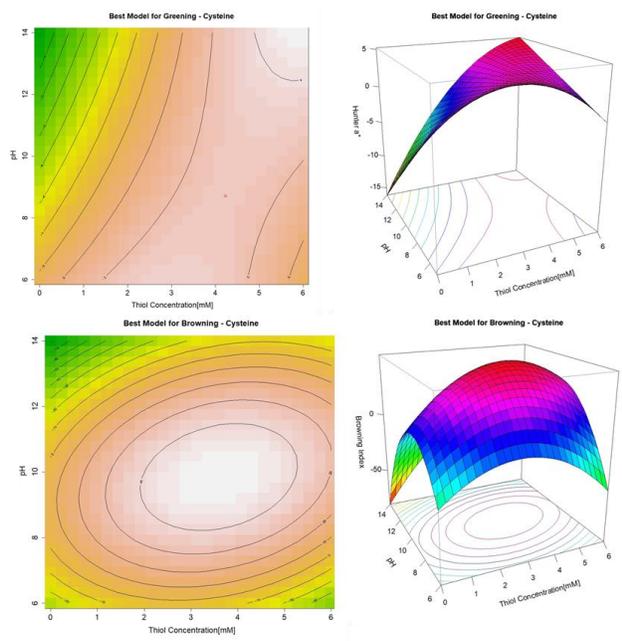


Figure 7 The contour plot of **greening** (represented by Hunter a*) and **browning index** of sunflower protein concentrate and isolates, measured after extraction and lyophilization (month-0/initial time point) as a function of extraction pH and thiol concentration of (A) cysteine and (B) glutathione based on Response Surface Methodology modeling from observed values.

4.3.2. Effect of thiol type and concentration on Hunter a* and Browning Index

Greening (hunter a*) was highest in control (pH 8.93-9.3; 0 mM) for both cys and GSH powders. The addition of cysteine resulted in powders with significantly lower greening than control (Table 7). Three months of storage at room temperature did not change the greening values and trends among cysteine and GSH powders as the color was unchanged over three months of storage.

Table 7 Greening (Hunter a*) and browning index of sunflower protein concentrate and isolate as a function of isolation pH and cysteine hydrochloride concentration

pH ranges	6.96-7.02	7.58-7.71	7.59-7.69	8.93-9.3	9.08-9.35	8.98-9.21	10.41- 10.57	10.47-10.51	11
Cysteine HCl (mM)	2.8	0.82	4.8	0	2.8	5.6	0.82	4.8	2.8
Replicate 1									
Replicate 2									

A) Cysteine

Time (months)

Hunter a* (Greening)

0	0.84±0.05 ^a	1.34±0.04 ^a	2.05±0.68 ^a	-9.94±3.99 ^{ab}	2.21±1.73 ^a	3.30±1.66 ^a	-3.85±3.50 ^{ab}	1.85±1.34 ^a	-0.90±0.59 ^{ab}
1	0.99±0.11 ^a	1.29±0.04 ^a	1.77±0.51 ^a	-8.15±1.62 ^{ab}	1.87±1.05 ^a	2.86±0.32 ^a	-2.58±1.03 ^{ab}	1.58±0.80 ^a	-0.95±0.91 ^{ab}
2	1.11±0.11 ^a	1.46±0.12 ^a	1.28±1.46 ^a	-8.74±0.86 ^{ab}	2.12±1.26 ^a	3.20±0.45 ^a	-2.96±0.85 ^{ab}	1.78±0.95 ^a	-1.07±0.90 ^{ab}
3	1.27±0.103 ^a	1.70±0.08 ^a	1.31±1.58 ^a	-9.05±0.44 ^{ab}	2.26±1.22 ^a	3.48±0.45 ^a	-3.16±0.88 ^d	1.82±0.98 ^a	-1.01±0.88 ^{ab}

Browning Index

0	19.25±2.65 ^a	23.59±1.88 ^a	21.44±3.15 ^a	-1.88±6.20 ^b	47.5±27.91 ^a	31.53±0.21 ^a	27.33±2.94 ^a	46.3±1.47 ^a	32.58±9.48 ^a
1	20.12±2.30 ^a	26.60±6.13 ^a	25.38±3.50 ^a	-1.35±8.11 ^b	47.37±24.50 ^a	38.07±9.19 ^a	28.36±12.76 ^a	51.86±10.70 ^a	35.50±16.96 ^a
2	19.23±1.59 ^a	23.88±4.07 ^a	18.30±1.91 ^a	-2.14±6.72 ^b	44.16±22.98 ^a	35.28±4.89 ^a	26.77±13.75 ^a	47.67±11.41 ^a	32.44±13.41 ^a
3	20.23±3.09 ^a	23.96±2.08 ^a	19.06±2.36 ^a	-2.02±6.19 ^b	43.96±22.74 ^a	35.87±7.25 ^a	24.49±11.85 ^a	45.90±9.16 ^a	32.95±14.00 ^a

Greening in powders with GSH was in general higher than those with cysteine, as powders with GSH ranged from -14.14 to 1.84, while powders with cys ranged from -8.74 to 4.47 (Table 8). The control (pH 9 – 9.30+0 mM GSH) and pH 10.41+0.82 mM GSH had the highest greening amongst all GSH treatments. A higher concentration (>0.82 mM) of GSH is needed compared to cysteine at higher pH (>10.41) to achieve a significant greening inhibition compared to that in control powders.

Table 8 Greening (Hunter a*) and browning index of sunflower protein concentrate and isolate as a function of isolation pH and GSH concentration (mM)

pH ranges	6.96-7.02	7.58-7.71	7.59-7.69	8.93-9.3	9.08-9.35	8.98-9.21	10.41-10.57	10.47-10.51	10.83-11
GSH (mM)	2.8	0.82	4.8	0	2.8	5.6	0.82	4.8	2.8
Replicate 1									
Replicate 2									
Time (months)									
Hunter a* over Time									
0	-0.52±0.49 ^a	-0.59±2.59 ^a	-0.54±0.17 ^a	-13.74±3.05 ^b	-0.76±0.82 ^a	0.22±0.64 ^a	-12.62±2.16 ^b	0.20±0.14 ^a	-1.67±2.14 ^a
1	-0.19±0.34 ^a	-0.37±1.17 ^a	-0.38±0.17 ^a	-10.17±1.60 ^b	-0.45±0.79 ^a	0.28±0.75 ^a	-10.04±0.20 ^b	0.50±0.49 ^a	-0.44±0.91 ^a
2	-0.09±0.16 ^a	-0.36±1.15 ^a	0.66±1.51 ^a	-11.10±1.75 ^b	-0.46±0.99 ^a	0.29±0.81 ^a	-10.96±0.06 ^b	0.63±0.82 ^a	-1.51±2.43 ^a
3	-0.26±0.11 ^a	-0.38±1.24 ^a	0.67±1.66 ^a	-11.93±2.38 ^b	-0.47±0.98 ^a	0.41±0.57 ^a	-11.68±1.22 ^b	0.78±0.65 ^a	-1.93±3.18 ^a
Browning Index									
0	12.39±0.07 ^a	15.77±4.17 ^a	14.26±1.61 ^a	-8.74±6.87 ^b	20.35±8.31 ^a	21.69±2.09 ^a	-12.43±3.21 ^b	26.11±7.35 ^a	18.95±11.2 ^a
1	13.46±1.05 ^b	15.87±3.42 ^{ab}	14.88±1.80 ^a	-9.53±8.15 ^b	19.25±7.52 ^a	22.52±3.37 ^a	-13.79±5.24 ^b	27.35±7.21 ^a	20.95±10.9 ^a
2	12.47±0.50 ^a	14.58±2.23 ^a	18.52±9.78 ^a	-9.77±7.46 ^b	19.15±8.21 ^a	21.21±2.99 ^a	-13.57±4.74 ^b	25.56±8.77 ^a	19.36±11.1 ^a
3	12.44±0.28 ^a	15.37±3.22 ^a	18.47±10.17 ^a	-9.62±7.33 ^b	17.55±6.03 ^a	20.54±2.77 ^a	-13.72±3.54 ^b	23.41±6.42 ^a	18.42±12.4 ^a

Compared with GSH, cysteine’s effectiveness in inhibiting greening could also be due to its higher Maillard browning. The same substrates are used for both reactions as the nucleophilic amine groups in cysteine and GSH might conjugate with carbonyls (reducing sugars and CGA quinone), resulting in yellow and brown pigments, as seen in Table 7 and 8 (Edwards & Wedzicha, 1997; Seong-Il, Eun-Jung, Ok-Hwan, & Boo-Yong, 2010). Over time, Maillard products can form in powders and foods that incorporate cysteine and GSH ingredients (Li, Jongberg, Andersen, Davies, & Lund, 2016; Lund et al., 2017). However, there were no significant differences in the powders' color over three months of storage in the current study. Though the color was not affected by time (three months of analysis), browning and greening were significantly affected by concentration and type of thiols (Figure 8). Overall, more brown pigments were formed in powders with cysteine than powders with GSH (48.51% higher BI) (Table 7 and 9), which could also be due to different rates and mechanisms of Maillard browning inhibition.

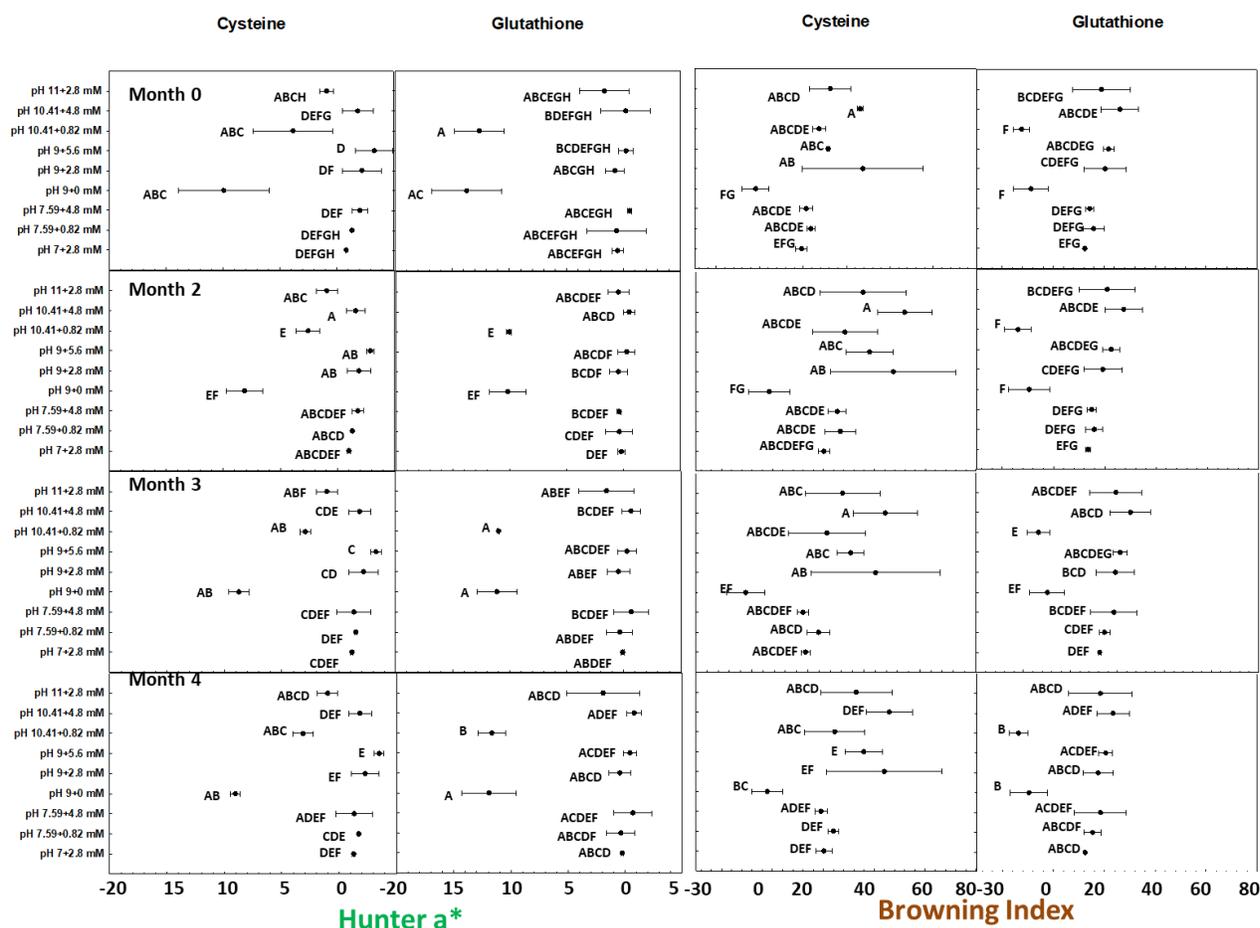


Figure 8 Kruskal Wallis test of greening (hunter a*) and browning index of cysteine and GSH per month. The same letters across row of greening and browning indicate no significant difference among treatments.

Glutathione inhibition of Maillard browning (higher hunter L*) has been reported in cookies (Zhu et al., 2020) due to GSH's N-terminal from glutamic acid side chain interacting with carbonyl groups reducing sugars in powders (Nomi, Aizawa, Kurata, Shindo, & Chuyen Van, 2009). Maillard browning can be inhibited with GSH during the propagation stage after Amadori products have formed. In contrast, cysteine inhibits Maillard browning at the initiation stage through direct competition with aldehydes via nucleophilic attack by thiol, and only if Amadori products have not been produced (Lu et al., 2019). Hence, cysteine inhibition is limited

only to the initial stages of the reaction, whereas GSH can react at the propagation stage resulting in the higher browning in powders with cysteine. Our results are also consistent with findings by Seong-II et al., (2010), who found that cysteine anti-browning capacity decreased faster than GSH in glucose-glutamic acid solutions stored at 30°C, and by (Li, Jongberg, Andersen, Davies, & Lund, 2016), who found that rate constant for the reaction of L-cysteine and GSH with 4-methyl-benzoquinone in proteins was higher for cysteine, which produced colored adducts. Overall, while cysteine was a more powerful greening inhibitor than GSH as a lower amount (0.82 mM) was needed to inhibit greening at higher pH (>10), powders with cysteine had more browning than GSH at alkaline pH.

4.4. Analysis of secondary and tertiary structure modification

4.4.1. Circular Dichroism

4.4.1.1. Effect of pH

Circular dichroism of sunflower protein powders revealed changes in protein secondary structure as a function of pH, thiol type, and concentration Figure 9 Far UV CD spectra of sunflower proteins as a function of thiol concentration of cysteine (left) and GSH (right) at pH ranges of 7-11 (Figure 9). Increasing pH from pH 7 to 11 in powders with 0.82 mM and 4.8 mM cysteine and glutathione induced alpha-helical shift into beta sheets (Figure 9) as seen from negative bands at 218, 208, and 222 nm and positive band at 195 nm (Mudasir Ahmad Malik & Saini, 2018). Regardless of concentration and thiol type, increasing pH from 9 to 11 resulted in alpha-helix to beta sheets conformational shift, which suggested protein unfolding caused by higher pH (>9), as helianthinin dissociation from 11S to 7S form is promoted (Gonzalez-Perez et al., 2004; Molina et al., 2004).

4.4.1.2.Effect of thiol type and concentration

They were increasing conformational shifts from α helical to β sheets as a function of adding thiols at neutral to pH 9 (Figure 9). A concentration-dependent alpha-helical to beta-sheets shift was detected with 0.82-2.8mM cysteine in pH 7 and pH 9 powders (Figure 9A and B). At higher Cys (4.8 and 5.6 mM) concentrations, proteins appeared to be in beta sheets regardless of pH, suggesting a higher Cys content induced protein unfolding. In contrast, there was no concentration protein unfolding effect of GSH at pH 9 and 11.

In general, secondary structure modification was induced by the combined effect of high pH, and thiol concentration as both higher thiol content of 4.8mM at pH 7 or high pH (>10) regardless of thiol concentration decreased α -helices.

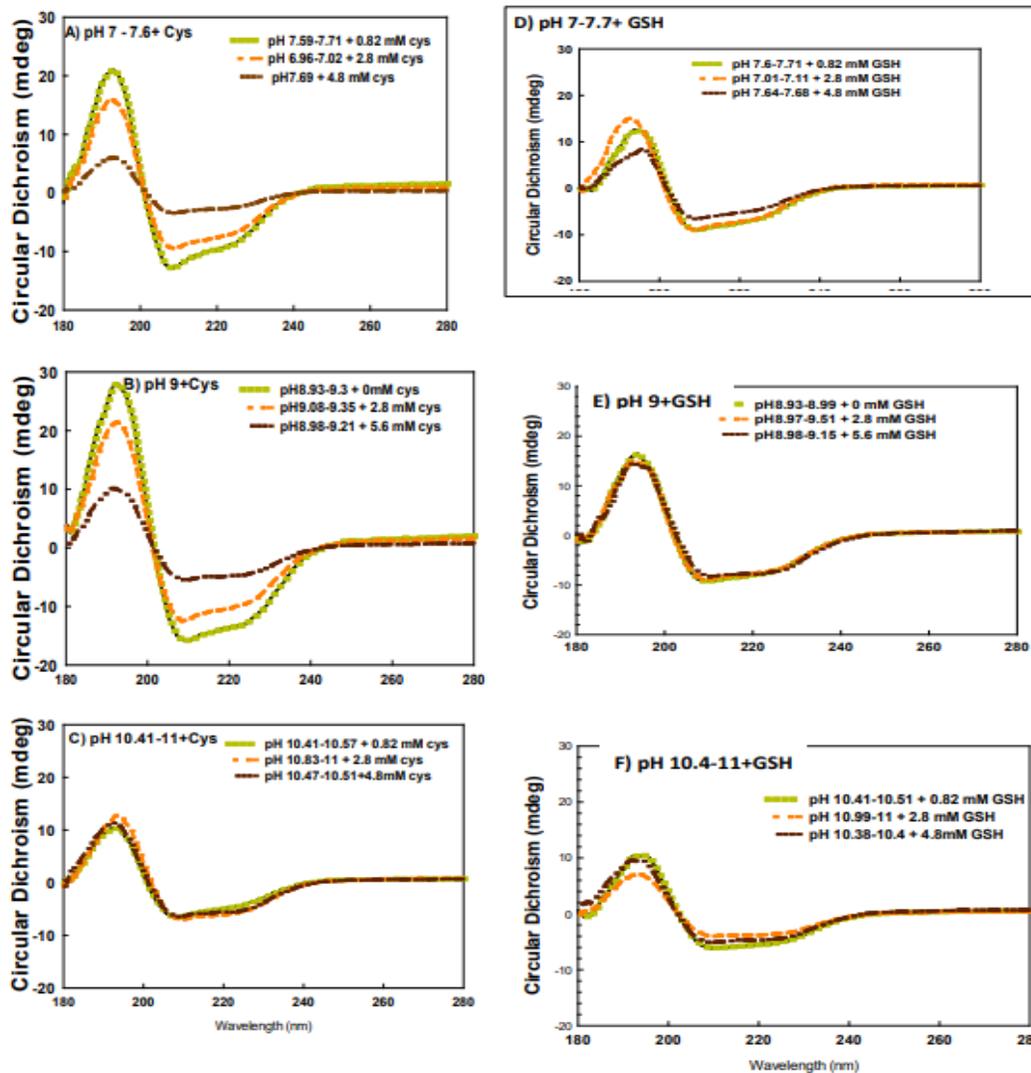


Figure 9 Far UV CD spectra of sunflower proteins as a function of thiol concentration of cysteine (left) and GSH (right) at pH ranges of 7-11

4.4.2. Fourier Transform Infrared Spectroscopy

Changes in absorbance in the amide I (1600 – 1700 nm), amide II (1500 – 1600 nm), and 1410 – 1310 nm regions correlated with protein-phenol conformational changes (Karefyllakis et al., 2018; Malik & Saini, 2018; Zhao, Mu, Zhang, & Richel, 2019). We detected absorbance peaks at amide 1 (1700-1600 nm), amide 2 (1580-1480 nm), 1410 – 1310 nm (N-H bending), and 3250-3300 nm regions (O-H stretching) at extraction pH of 7-11 (Fig. 6.). However, there were no thiol induced secondary structure modifications attributed to protein-thiol conjugation or

prevention of CGA-protein conjugation. This was in contrast to D. Karefyllakis et al. (2018) and Zhao et al., 2019) who found that both CGA-protein and thiol-protein complexation can induce unfolding and decrease absorbance in amide 1 and 2 regions.

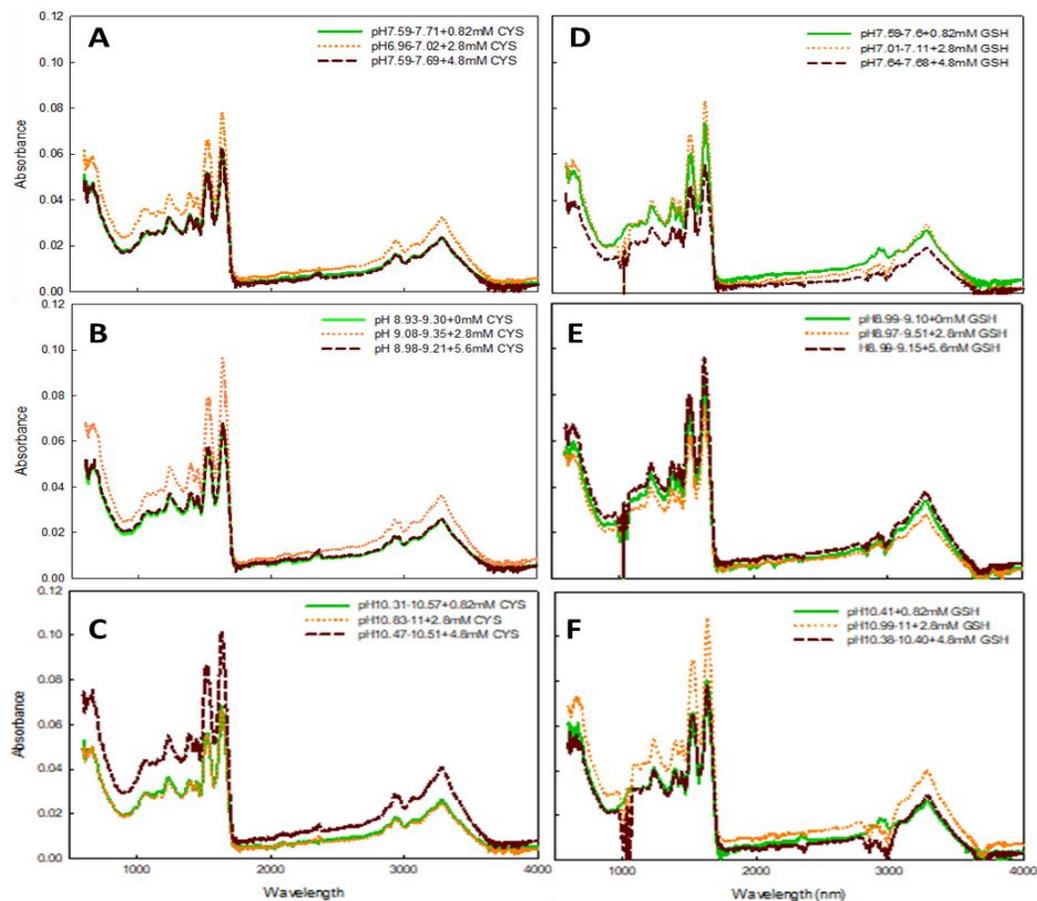


Figure 10 Fourier Transform Infrared Spectroscopy of sunflower protein concentrate and isolate as a function of both pH (7-11) and 0-5.6 mM thiol concentration (cysteine-A-C; GSH-D-F) after lyophilized and ground (0 month storage)

4.4.3. Intrinsic Tryptophan Fluorescence as a function of pH and thiols

The maximum emission wavelength of solubilized protein powders ranged from 354 to 361 nm, which was close to the expected maximum λ_{em} of tryptophan (360 nm) that was observed in sunflower butter cookie dough (Atonfack et al., 2019) and in 0.1 M phosphate buffer, pH 7 (Kirby & Steiner, 1970). Regardless of thiol type and concentration, increasing pH resulted

in fluorescence quenching (Figure 11), as samples extracted around pH 7 exhibited the highest intensity, followed by pH 9 and 11. Our results are consistent with previous findings from Sripad & Rao (1987), where alkaline pH changes the tertiary protein conformation of helianthinin by modifying the overall protein charge and electrostatic interactions, exposing the previously buried tryptophan.

Alkaline pH causes conjugation of oxidized CGA quinones and sunflower protein, which induces fluorescence quenching at 300-500 nm regions and causes irreversible changes to the tertiary structure, decreasing polarity (D. Karefyllakis et al., 2018). Nonetheless, cysteine addition during alkaline pH (9-11, Figure 11A) extraction increased fluorescence intensity in a concentration-dependent manner, suggesting that the CGA-protein complex was prevented. Our results are consistent with Nielsen, Lund, Davies, Nielsen, & Nielsen, (2018), who reported that cysteine addition mitigated unfolding in whey protein when exposed to 90°C, thus increasing fluorescence intensity and allowing the formation of unfolded protein aggregates (Nielsen et al., 2018). While the concentration-dependent decrease of alpha-helices in pH 7 and 9 cys' powder solutions was observed with CD (Figure 9), fluorescence spectroscopy confirmed that at alkaline pH, cysteine could induce protein refolding at a high concentration as pH 9 powder solutions with 2.8 and 5.6 mM; pH 11 with 4.8 mM had higher fluorescence intensities than control (Figure 11A).

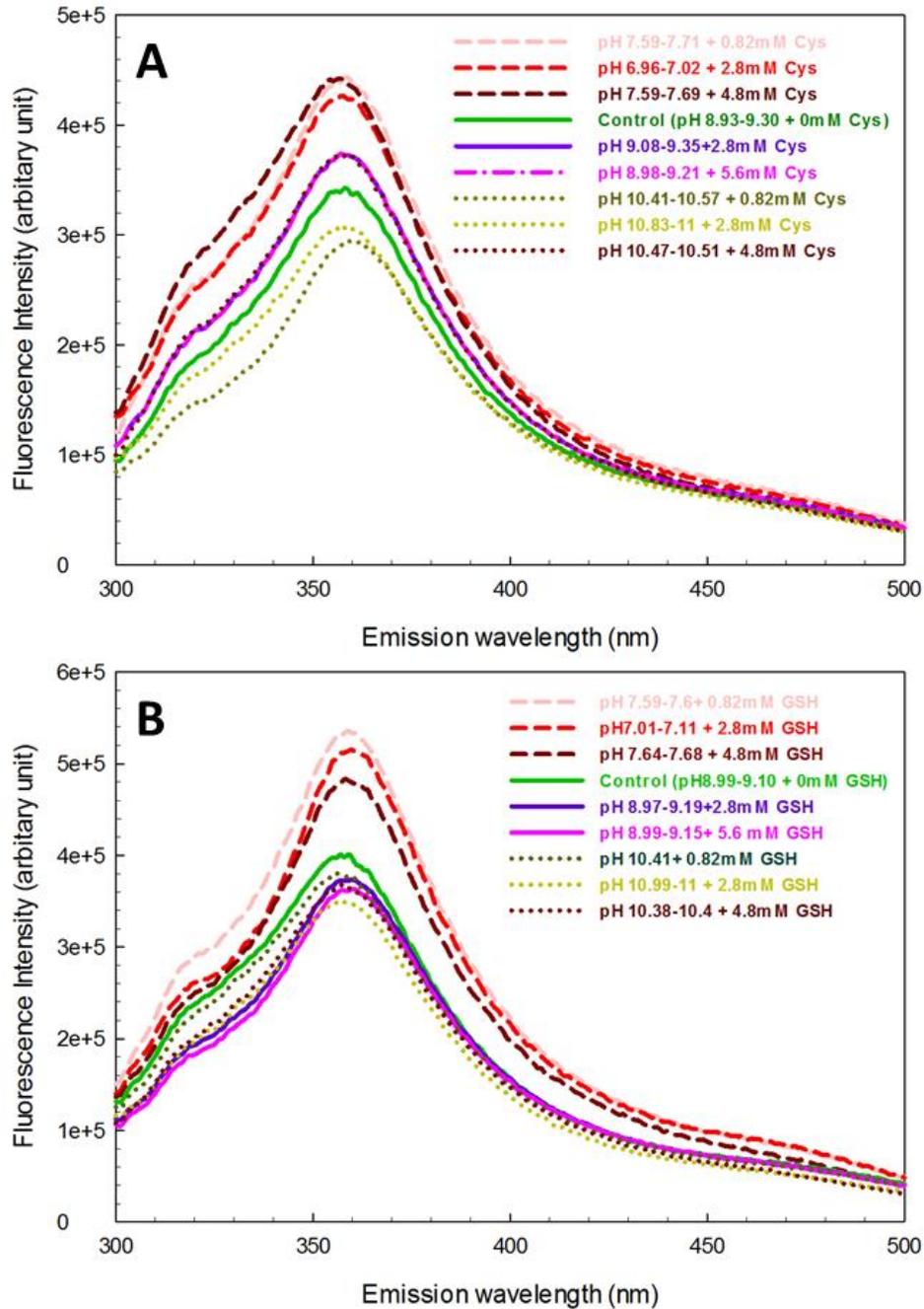


Figure 11 Fluorescence Intensity of solubilized sunflower protein, extracted with Cysteine HCl (A) and GSH (B), and extraction pH of 7 (--dashed line), 9 (-straight line), and 11 (..dotted line)

Higher fluorescence intensity was observed in powders with cysteine, which also had higher browning than powders with GSH (Table 7 and 8; Lu et al., 2019; Villanova et al., 2017). Thus,

cysteine prevention of CGA-protein conjugates could also result in CGA-thiol conjugates' formation, which could also induce browning (Z. Zhang et al., 2018). Nevertheless, concentration-dependent fluorescence quenching was detected with GSH at all extracted pHs, which was the opposite of cysteine. Jahanban-Esfahlan & Panahi-Azar, (2016) reported that GSH addition in bovine serum albumin caused GSH-protein complexation, which quenched fluorescence. As a result, GSH thiols are less available to conjugates with CGA quinones. Thus, GSH powders are greener than cysteine (Table 7 and 8), and less soluble CGA was detected with higher GSH at pH 9 (Figure 5), which could be due to more CGA oxidations and formations of green TBA.

5. Conclusions

Protein concentration was not affected by the addition of thiols. Adding 2.8 mM thiols resulted in the highest soluble CGA than 0 (control) or 5.6 mM, which suggested that CGA-protein conjugate occurred no added thiol ingredients, while CGA-thiol conjugates increased with higher thiol concentrations. SDS-PAGE analysis confirmed that higher pH (10.40) and thiol concentration (4.8 mM) caused 11S helianthinin dissociation into 7S form with cysteine, but the 11S form can be retained with GSH. Adding either Cysteine HCl or GSH prevented greening during alkaline extraction of sunflower protein, but each thiol has its benefits and disadvantages. A higher concentration (>0.82 mM) is needed with GSH at higher pH (>10.40) to achieve significant greening inhibition. The use of cysteine however resulted in powders with more non-enzymatic browning than GSH. FTIR and CD analysis suggested that at alkaline pH, cysteine addition caused secondary structure modification through a decrease in alpha helices, while GSH did not modify protein structure. However, fluorescence spectroscopy indicated that cysteine could induce protein refolding when exposed to alkaline pH, whereas GSH induces fluorescence

quenching. As cysteine or GSH incorporation into alkaline sunflower protein extraction will not require de-phenolization, adding thiol should result in sunflower protein with minimal greening.

6. Recommendations for future studies

Alkaline protein extraction of sunflower ingredients with thiol ingredients to lower greening through CGA-thiol conjugation has not been studied. Since this was the first study to examine the effects of thiol in alkaline extraction, we used Heliator® sunflower flour due to retention of polyphenol (Austrade). Future experiments could start with less processed ingredients (meal and seeds) and utilizing RSM modeling to model greening inhibition at alkaline pH. The effect of thiol additions should be further investigated to evaluate changes in proteins' functionalities while maintaining a high extraction yield. Moreover, a sensory analysis should be conducted to study consumer acceptance of sunflower protein powders' flavor when incorporated in baking.

7. References

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8. Appendix

Sunflower protein extraction dataset

Changes in pH for every 10 mins during protein extraction, after sunflower flour, had solubilized, and targeted pH had been reached using NaOH.

Table 9 pH adjustment during sunflower protein extraction with Cysteine HCl

Replicate	Target pH	Cysteine HCl (mM)	pH for time point measurement				Isoelectric pH
			0 min	10 min	20 min	30 min (final pH)	
1	7.59	0.82	7.6	7.75	7.6	7.58	5.61
	10.41	0.82	10.49	10.41	10.41	10.41	5.59
	9	2.8	9.06	8	8.95	9.35	5.59
	7.59	4.8	7.7	7.58	7.6	7.69	5.61
	10.41	4.8	8.98	10.41	10.41	10.47	5.19
	9	5.6	9	9	8.98	9.21	5.47
	9	0	8.99	9.12	9.2	9.3	5.19
	11	2.8	10.59	10.88	10.95	11	5.25
	9	2.8	8.93	8.99	9.1	9.19	5.59
	7	2.8	6.99	7.01	7.01	7.02	5.58
2	7.59	0.82	7.57	7.61	7.66	7.71	5.58
	10.41	0.82	10.38	10.42	10.38	10.57	4.31
	9	2.8	8.99	9.05	9.06	9.08	5.48
	7.59	4.8	7.58	7.61	7.57	7.59	5.58
	10.41	4.8	10.49	10.47	10.51	10.51	5.58
	9	5.6	9	8.97	8.92	8.98	5.64
	9	0	8.96	8.96	8.9	8.93	5.43
	11	2.8	10.98	10.78	10.9	10.83	5.42
	9	2.8	9.05	9.27	0.19	9.19	5.59
	7	2.8	6.96	7.01	6.99	6.96	4.64

Below is the record for changes in pH for every 10 mins during protein extraction, after sunflower flour was solubilized and targeted pH had been reached after reduced glutathione has been solubilized. NaOH is added to reach target pH.

Table 10 pH adjustment during sunflower protein extraction with glutathione

Replicate	Target pH	GSH Concentration	pH for time point measurement				Isoelectric pH
			0 min	10 min	20 min	30 min (final pH)	
1	7.59	0.82	7.63	7.59	7.58	7.58	5.59
	10.41	0.82	10.41	10.38	10.4	10.41	5.59
	9	2.8	9.64	9.55	9.56	9.35	5.48
	7.59	4.8	7.6	7.66	7.64	7.69	5.52
	10.41	4.8	10.38	10.39	10.44	10.47	5.58
	9	5.6	9.04	9.17	9.18	9.21	4.88
	9	0	9.17	9.21	9.26	9.3	5.55
	11	2.8	10.61	10.95	10.98	11	5.61
	9	2.8	9.15	9.39	9.31	9.19	5.39
	7	2.8	7.05	7	7.02	7.02	5.51
2	7.59	0.82	7.8	7.79	7.65	7.71	5.58
	10.41	0.82	10.42	10.43	10.25	10.57	4.31
	9	2.8	9.03	9.05	9.07	9.08	5.48
	7.59	4.8	7.57	7.58	7.59	7.59	5.58
	10.41	4.8	10.47	10.49	10.5	10.51	5.58
	9	5.6	8.99	8.99	8.98	8.98	5.64
	9	0	8.88	8.96	8.94	8.93	5.43
	11	2.8	10.99	10.86	10.95	10.83	5.42
	9	2.8	9.1	9.14	9.18	9.19	5.59
	7	2.8	7.01	6.96	6.96	6.96	4.64

Chlorogenic acid identification with LC-MS (Ishii, Liang, & Were, 2020)

In the preliminary experiment of sunflower protein prepared with pH 8.3 buffered solution, a compound with high abundance at 11.2 mins was identified as L-lys-quinone adduct, a conjugate product of o-benzoquinone with lysine (Li et al., 2020). This adduct eluted at 11.2 mins for both 0 mM, and when 5.6 mM cysteine was added (Table 11). The relative intensity was lower with added cysteine, which could indicate that cysteine-phenol conjugate occurred as the thiol group from cysteine is much more nucleophilic than lysine (Verena Bongartz et al., 2016). A high abundance of CGA was also detected at 12.3 mins elution time for both control and with 5.6 mM cysteine added. Through the addition of cysteine, cysteine-CGA conjugate in the form of 2'-S-cysteinyl-5-

O-caffeoylquinic acid was identified at the retention time 11.2, 11.3, and 11.8 mins (Kuijpers et al., 2012).

Table 11 Identification of reaction products of CGA and Cysteine in H55 + 5.6 mM cysteine solutions after alkaline extraction (pH 8.3) and isoelectric point (pH = 5.6) precipitation.

Compound	Retention time (min)	[M+H] ⁺	MS fragments (abundance)	(%relative abundance)	Tentative identification
1	1.9	663	115(45), 130 (41), 156 (30), 200 (100), 201 (14), 248 (44), 338 (19), 551 (41), 552 (17), 663 (23)		
2	2.0	664	115 (62), 130 (48), 156 (41), 200 (100), 248 (51), 338 (23), 551 (57), 552 (24), 663 (31), 664 (17)		
3	3.8	248	74 (26), 83 (28), 85 (15), 91 (15), 110 (45), 122 (21), 126 (21), 237 (17), 239 (17), 248 (100)		
4	4.5	703	91 (55), 159 (36), 227 (100), 243 (57), 248 (49), 363 (27), 431 (34), 447 (21), 567 (21), 703 (20)		
5	4.9	248	91 (33), 130 (18), 153 (18), 159 (21), 227 (42), 237 (18), 239 (17), 241 (27), 243 (24), 248 (100)		
6	11.2	476	86 (8), 130 (5), 132 (8), 207 (9), 266 (100), 267 (16), 268 (8), 387 (6), 474 (7), 476 (9)		L-lysine quinone adduct (268), 2'-S-cysteinyl-5-O-caffeoylquinic acid conjugate (474)
7	11.3	1161	188 (3), 207 (8), 248 (4), 266 (100), 267 (15), 268 (3), 474 (4), 476 (3), 1160 (3), 1161 (3)		
8	11.8	474	130 (2), 163 (3), 188 (8), 205 (6), 207 (8), 248 (5), 266 (100), 267 (16), 268 (2), 474 (2)		2'-S-cysteinyl-5-O-caffeoylquinic acid conjugate (474)
9	12.3	731	122 (6), 130 (3), 163 (100), 164 (10), 248 (14), 266 (10), 355 (67), 356 (12), 377 (3), 731 (4)		Chlorogenic acid (355),