



Preventing chlorogenic acid quinone-induced greening in sunflower cookies by chlorogenic acid esterase and thiol-based dough conditioners

Nana Baah Pepra-Ameyaw, Christine Lo Verde, Charles T. Drucker, Cedric P. Owens, Lilian W. Senger*

Schmid College of Science and Technology, Chapman University One University Drive, Orange, CA, 92866, USA

ARTICLE INFO

Keywords:

Chlorogenic acid esterase
Cysteine
Caffeic acid
Glutathione
Sunflower butter
Sunflower flour

ABSTRACT

Sunflower seeds contain a high concentration of chlorogenic acid (CGA), which reacts with amino acids to form green pigments under alkaline conditions during food processing. Here, we present two approaches to prevent green pigment formation in sunflower cookies by (A) Addition of free thiols from cysteine and glutathione to sunflower cookie dough and (B) hydrolyzing CGA into caffeic acid and quinic acid with a CGA esterase from *Lactobacillus helveticus*. Greening occurred more slowly with cysteine; however, neither cysteine nor glutathione prevented greening in the cookies during storage. Chlorogenic acid esterase hydrolyzed CGA in both sunflower butter and flour, resulting in the complete elimination of greening in the sunflower cookies. CGA esterase treatment was efficient as the enzyme could be applied in low amounts (<100 ppm) directly to the dough without needing to pretreat either sunflower butter or flour. Overall, our data indicate that CGA esterase treatment was an effective method of eliminating unwanted greening in sunflower cookies made with baking soda. Long term, these results may represent a method of increasing the use of sunflower butter and flour in high pH baking applications by enabling their use in neutrally colored baked products such as cookies and muffins.

1. Introduction

The most prevalent phenolic compound in sunflower seeds is the hydroxycinnamic acid derivative, chlorogenic acid (CGA), which is an ester of caffeic acid (CA) and quinic acid (QA) (de Oliveira Filho & Egea, 2021). The presence of CGA in sunflower seeds gives rise to a green pigment during processing under alkaline conditions (Bongartz et al., 2016; Namiki, Yabuta, Koizumi, & Yano, 2001; Yabuta, Koizumi, Namiki, Hida, & Namiki, 2001). The formation of the green pigment occurs when the catechol moiety of CGA is oxidized to form an o-quinone intermediate, which is electron-deficient and readily reacts with nucleophilic amines in proteins (Wildermuth, Young, & Were, 2016).

CGA quinone-induced color formation can hinder the use of sunflower butter and sunflower flour in consumer food products (Adeleke & Babalola, 2020; de Oliveira Filho & Egea, 2021). Sunflower is primarily produced as an economically important oilseed crop, with approximately 60% of sunflowers being produced globally for oil production. The sunflower meal, a byproduct obtained after oil production, is used

for animal feed, fertilizer, or for energy production (Adeleke & Babalola, 2020; Soare & Chiurciu, 2018). Prevention of CGA quinone-induced green color formation could add value to sunflower seed-derived ingredients and increase their use in consumer food products, for example, by milling the meal into flour for baking.

Measures to mitigate or prevent green color formation during sunflower food production have included solvent-based CGA extraction and filtration; however, these processes remove beneficial phenolic compounds, may not be eco-friendly, and are costly (González-Pérez et al., 2002; Pickardt, Eisner, Kammerer, & Carle, 2015; Wianowska & Gil, 2019). Thiol addition is a promising method of greening prevention that retains CGA. Thiol-containing cysteine and glutathione have been shown to limit greening in model CGA-lysine solutions and in alkaline-processed sunflower protein isolates (Drucker, Senger, & Pacioles, 2023; Ishii, Toto Pacioles, & Were, 2021; Liang & Were, 2020). Thiol compounds are hypothesized to prevent CGA greening reactions by forming colorless thiolyl-CGA conjugates as deprotonated thiolate anions react with quinones more readily than amine nucleophiles (Bongartz et al., 2016). Thiols act as reducing dough conditioners,

* Corresponding author. Schmid College of Science and Technology, Chapman University One University Drive, Orange, CA, 92866, USA.

E-mail addresses: pepraame@chapman.edu (N.B. Pepra-Ameyaw), cloverde@chapman.edu (C. Lo Verde), cdrucker@chapman.edu (C.T. Drucker), cpowens@chapman.edu (C.P. Owens), were@chapman.edu (L.W. Senger).

<https://doi.org/10.1016/j.lwt.2022.114392>

Received 18 July 2022; Received in revised form 24 December 2022; Accepted 27 December 2022

Available online 28 December 2022

0023-6438/© 2022 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

thereby influencing the rheological properties of dough formulations (Tozatti et al., 2020). No work has been done, however, to investigate the thiol effect on greening mitigation in baked foods that contain CGA and high pH ingredients. In the United States, L-cysteine (henceforth referred to as cysteine) is approved for use as a food ingredient up to 90 ppm of flour in dough, yeast-leavened baked goods, and baking mixes (21CFR184.1271). The European Food Safety Authority (EFSA) permits its use in flour up to *quantum satis* by the commission regulation (EU) No231/2012 (EU, 2012). Glutathione is used as a generally recognized as safe (GRAS) food ingredient and supplement. Given the preliminary evidence that cysteine and glutathione limit green pigment formation and that both thiol-containing compounds are safe to use in foods, we hypothesized that cysteine and glutathione would prevent greening in sunflower butter (SFB) cookies. Our group previously investigated the formation of green color in SFB cookies when cysteine was incorporated at levels up to 90 ppm (0.09%) and found no mitigation of green color formation in the cookies (Liang & Were, 2020). Therefore, in the present study, we evaluated cysteine and glutathione at higher concentrations.

An alternative approach to greening prevention by thiol treatment is CGA hydrolysis by esterases. Esterases are part of the hydrolase class of enzymes, which are widely used in food processing applications, including in the baking industry (De Vos et al., 2006; Frauenlob, Scharl, S., & Schoenlechner, 2018; Sathya & Khan, 2014) and are considered “clean label” (Dai & Tyl, 2021). Chlorogenic acid esterases cleave CGA into caffeic and quinic and have been isolated from both fungal and bacterial sources (Benoit et al., 2007; Esteban-Torres et al., 2015; Lai, Lorca, & Gonzalez, 2009). Chlorogenic acid esterases have been used for hydrolysis of hydroxycinnamic acid derivatives in coffee, wheat, and rapeseed (Bel-Rhliid, Thapa, Kraehenbuehl, Hansen, & Fischer, 2013; Kraehenbuehl et al., 2017; Laguna et al., 2019; Siebert, Berger, & Nieter, 2018). A selection of potential applications of CGA esterases in the food and beverage industry are listed in Table 1. Notably, CGA cleavage by fungal esterases was evaluated by Zhang, Liu, Hu, and Yang (2019) as a method to prevent greening in sunflower protein isolates. However, the study was inconclusive since isolates were kept under acidic conditions throughout the experiment, which lowers greening regardless of

whether CGA is hydrolyzed or not. A follow-up study by Zhang, Wang, Liu, and Leng (2021) in alkaline conditions was also inconclusive since CGA was extracted before esterase treatment, and thus the effectiveness of enzymatic CGA hydrolysis-based greening prevention could not be assessed.

While both fungal and bacterial esterases hydrolyze CGA, bacterial CGA esterases are considerably more active (Asther et al., 2005; Song & Baik, 2017). Our group recently characterized a CGA esterase from *Lactobacillus helveticus* that has a K_m of 0.090 mM and a k_{cat} of 82 s^{-1} and was shown to be stable over a wide range of pH and temperatures (Lo Verde et al., 2022). We further demonstrated that *L. helveticus* CGA esterase (henceforth simply referred to as CGA esterase) rapidly hydrolyzed CGA in sunflower meals and fully eliminated greening in alkaline extracted protein isolates. These results represented the first time non-greening sunflower protein isolates could be produced under alkaline conditions without any prior processing steps such as dephenolization.

Since the CGA esterase was active in both defatted and undefatted sunflower meals and was shown to cleave CGA in both dilute solutions and sunflower meal suspensions, we hypothesized that CGA esterase would be able to cleave CGA in both sunflower flour and butter. The research objective was thus to compare the prevention of greening of thiols to that of CGA esterase, with the goal of enabling the production of cookies made with sunflower flour (SFF) and SFB that do not turn green.

2. Materials and methods

2.1. Materials

Sunflower kernels (Lyric Wild Bird Food, PA, USA) were obtained from Home Depot (CA, USA). CGA esterase was purified as described previously (Lo Verde et al., 2022). For all experiments, CGA esterase was buffered in 50 mM HEPES, pH = 8.0, unless otherwise specified. All buffers utilized for experiments were comprised of 50 mM HEPES, pH = 8.0. Cysteine (CAS: 52-90-4) was obtained from Sigma-Aldrich (MA, USA) and glutathione (CAS: 70-18-8) from Acros Organics (NJ, USA). All-purpose flour (UPC: 016000106109, First Street, CA, USA), sunflower seed butter (Once Again unsweetened and salt-free, UPC: 044082530413, NY, USA), fresh eggs, baking soda (UPC: 033200016700), vanilla extract (UPC: 089836185327) and salt (UPC: 041512005275), and maple syrup (UPC: 681170411201) (Kirkland, WA) were purchased from local grocery stores. All solvents and acids were analytical or HPLC grade (Fisher Scientific, PA, USA).

2.2. Cookie formulations and baking conditions

The cookie dough formulation consisted of baking soda (2.2 g), salt (2.2 g), whole egg (46.8 g), maple syrup (76.3 g), vanilla extract (2.2 g), flour (145.8 g), and butter (87.5 g). For cysteine, glutathione, and CGA esterase experiments using SFB, all-purpose flour was the flour source used. CGA esterase treatments in SFF, utilized almond butter instead of SFB. The wet (sunflower or almond butter, maple syrup, eggs, and vanilla extract) and dry (flour, baking soda, and salt) ingredients were mixed separately and subsequently combined and mixed for 75 s with an electric hand mixer (Cuisinart, CT, USA). The dough was rolled out and cut to the following dimensions: 6 mm thickness and 40 mm diameter. Cookies were baked for 7 min in a convection oven (Hobart Vulcan, MD, USA) preheated to 149 °C (300 °F) and allowed to cool at room temperature before further analysis.

2.3. Thiol-treatment of sunflower butter

Cysteine and glutathione were added to the sunflower butter at three addition levels: 0.0 (negative control), 0.5% (w/w), and 1.0% (w/w) thiol per g butter. The required amount of thiol was weighed and mixed into 87.5 g of sunflower butter. The mixture was stirred for 1 min and

Table 1
Food and beverage applications of chlorogenic and ferulic acid esterases.

Enzyme	Enzyme source	Food tested	Effect	Reference
Chlorogenic acid esterase	<i>Ustilago maydis</i>	Wheat dough	Dough softening	Nieter et al. (2016)
Chlorogenic acid esterase	<i>Ustilago maydis</i>	Destarched wheat bran and coffee pulp	released ferulic, p-coumaric, and caffeic acids	Nieter et al. (2015)
p-coumaroyl esterase	<i>Rhizoctonia solani</i>	Coffee powder	No odor and taste changes	Siebert et al. (2018)
Hog liver esterases	<i>Sus scrofa</i>	Coffee extract	Debittered coffee	Kraehenbuehl et al. (2017)
Chlorogenic acid esterase	<i>Lactobacillus johnsonii</i>	Coffee extract	Complete hydrolysis of 5-CGA into caffeic acid	Bel-Rhliid et al. (2013)
Chlorogenic acid esterase	<i>Aspergillus niger</i>	Coffee pulp	Produced caffeic acid	Asther et al. (2005)
Chlorogenic acid esterase	<i>Lactobacillus helveticus</i>	Sunflower protein isolates	Complete hydrolysis of 5-CGA and greening inhibition	Lo Verde et al. (2022)
Chlorogenic acid esterase	<i>Aspergillus niger</i>	Sunflower seed protein	Greening prevention	Zhang et al. (2019)
Ferulic Acid Esterase	<i>Aspergillus aculeatus</i>	Sunflower seed protein	Greening prevention	Zhang et al. (2021)

allowed to stand for 10 min before mixing with other dough ingredients as described in section 2.2.

2.4. Preparation of sunflower butter cookies with chlorogenic acid/CGA esterase

Two concentrations of CGA esterase, 1.72×10^{-1} mg and 1.72×10^{-2} mg of enzyme per g of sunflower butter, were added to 97.48 g of sunflower butter. The volume of the enzyme was <3 mL. Control cookies were made by adding buffer instead of enzyme solution. The mixture was allowed to stand for 10 min and stirred for 1 min after 3 min and 7 min. Cookies were then prepared as described in section 2.2.

2.5. Preparation of sunflower flour cookies with CGA esterase

Sunflower flour was made by grinding sunflower seeds in a coffee grinder (Model BCG111, KitchenAid Blade MD, USA) for 1 min and sieved through a 500 μ m steel mesh. CGA esterase treatment of the flour occurred in two ways: pretreatment and direct treatment. For pretreated flour, flour was suspended in 50 mM HEPES buffer at pH = 8.0 before the addition of 0.09 mg CGA esterase per g of meal for 10 min. Following enzymatic treatment, sunflower flour was then lyophilized for 20 h as described by Ishii et al. (2021). The resulting flour was added to make cookies as described in section 2.2. For the direct treatment, sunflower seed flour was prepared similarly in 50 mM HEPES buffer at pH = 8.0 and lyophilized without the addition of the enzyme. The esterase (0.09 mg/g) was then added directly to the lyophilized sunflower flour during cookie dough preparation, as described in section 2.2.

2.6. Cookie diameter, height, and spread ratio measurement

The average diameter and height (mm) of sunflower cookies were measured using a digital caliper. The spread ratio was then calculated as the ratio of the average diameter to the average height as described by Handa, Goomer, and Siddhu (2012).

2.7. Moisture content and fat extraction

After baking, the moisture and crude fat content of the cookies were determined using AOAC Official Methods 934.06 and 948.22, respectively (AOAC, 2019). To determine the moisture content, approximately 5 g of the cookie samples were dried in a vacuum oven (Model 3618-5, Thermo Scientific, MA, USA) for 24 h at 70 °C at a pressure of 25 bar. Crude fat content was determined by Soxhlet extracting 4–5 g of the dried cookies samples for 16–24 h in hexane.

2.8. High-performance liquid chromatography (HPLC) quantification of phenolic content in sunflower cookies

Following Soxhlet fat extraction, 0.5 g samples of the defatted sunflower cookies were placed into 10 mL of 70% ethanol to extract phenolics for HPLC analysis. The extraction mixture was vortexed and allowed to stand for 1 h before centrifuging at 17,000 \times g for 15 min (accuSpin Micro 17 R, Fisher Scientific, Germany). The extract was filtered through 0.20 μ m syringe filters and analyzed with an Agilent 1260 Infinity II system (Agilent Technologies, Germany) equipped with a quaternary pump (G7111B), vial sampler (G7129A), and diode array detector (HS G7117C). The chromatographic separation was done through a Phenomenex Luna 5 μ m C18(2) 100 Å LC column (150 \times 4.6 mm) without controlling temperature. The samples were eluted using 0.1% formic acid in HPLC grade water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) as specified previously (Lo Verde et al., 2022). The separation was done at a flow rate of 1.5 mL min⁻¹ by increasing the gradient of solvent B from 13 to 23% in 6 min. CGA and caffeic acid concentrations were detected at 320 nm and quantified using a standard curve between 0.00 and 0.10 mg mL⁻¹ CGA ($y = 172287x$, $R^2 = 0.994$)

and caffeic acid ($y = 248557x$, $R^2 = 0.967$).

2.9. CIE L*a*b* determination of greening and browning

The color development in the cookies following baking was determined after 1, 3, and 24 h. The first time point was to ensure that cookies were at room temperature before any measurements were taken. At each time point, replicate cookies were cut in half along the diameter. The color was determined as CIE L*a*b* values using a CM-2500D spectrophotometer (Konica Minolta, Inc. Tokyo, Japan). The CIE L* values indicated the lightness of the cookie (White = 100; Black = 0), a* values indicated greening (positive = red; negative = green), and b* values indicated yellowness (positive = yellow; negative = blue). Browning index was determined as outlined by Ishii et al. (2021) using Eq. (1):

$$\text{Browning index} = \frac{(x - 0.31)}{0.172} \times 100. \quad (\text{Eq1})$$

where

$$x = \frac{(a + 1.75L)a}{(5.645L + a - 3.013b)}$$

2.10. Water activity and cookie pH measurement

Water activity (a_w) was determined on the cookies using a Series 3 Aqua Lab water activity meter (WA, USA) after 0, 1, 3, and 24 h following baking.

Cookie samples (0.5 g) were mixed in 5 mL of nanopure water and homogenized for 1 min at 15,000 \times g (Multi-prep Homogenizer, Pro-Scientific, CT, USA). The homogenized samples were incubated for 24 h at room temperature and centrifuged at 4,646 \times g for 30 min before measuring the pH of the supernatant (LabQuest 2 pH meter, Vernier Software & Technology, OR, USA).

2.11. Folin-Ciocalteu reducing capacity

The Folin-Ciocalteu reducing capacity in cookies treated with CGA esterase was determined after 1, 3, and 24 h following baking as described by (Singleton, R., & Rossi, 1965). One gram cookie samples were homogenized in nanopure water and centrifuged at 4,646 \times g for 30 min. A 100 μ L sample of supernatant was diluted to 3 mL with water and mixed with 0.5 mL of Folin-Ciocalteu reagent. After 5 min, 2 mL of 20% sodium carbonate was added, and the contents were vortexed and incubated at room temperature for 40 min. Absorbance was measured at 760 nm using CGA as a standard. The results were expressed as mg CGA/g of fresh weight material and analyzed by ANOVA with a significance level of $P < 0.05$.

2.12. Statistical analysis

Cookie spread ratio, moisture, and lipid content between treated and untreated cookies (cysteine, glutathione, and CGA esterase addition) were analyzed by analysis of variance (ANOVA) at the initial time point after baking. For each treatment type, the differences in greening (a^*) between treated cookies (cysteine, glutathione, and CGA esterase addition) and their corresponding untreated control over time (0, 1, 3, and 24 h) were analyzed by analysis of covariance (ANCOVA) to control for the known effects of pH and water activity, which were considered covariates, on greening. Statistical significance was determined at $P < 0.05$. Tukey's HSD test was used to distinguish between significant groups where appropriate. Statistical analyses were performed using R statistical software v. 4.1.2 (R Core Team, 2021). All measurements were expressed as the mean \pm standard deviation of independent duplicate determinations.

3. Results and discussion

3.1. Spread ratio, crude fat, moisture content, pH, and water activity of cookies

The spread ratio and water activity are factors that affect greening in cookies (Liang & Were, 2018). These parameters were measured in cookies that received thiol and enzyme treatment and in their corresponding untreated controls. Size, moisture, and lipid content did not differ significantly ($P > 0.05$) between treated and untreated cookies based on an analysis of variance (ANOVA). Thus, these variables were excluded when assessing how the respective treatments affect greening in cookies (Supplementary Material Table S1).

Two additional variables that affect cookie color are pH and water activity/ a_w (Liang & Were, 2018). Therefore, it was determined to what extent greening is influenced by pH, water activity, and thiol addition (Table 2). pH measurements showed that the pH differed between some treated vs. untreated cookies and therefore pH was included in subsequent models. Similarly, a_w changed as cookies were stored over time so a_w was included in the greening models (Supplementary Material Tables S2–S5).

3.2. Effect of cysteine and glutathione on greening in sunflower butter cookies

Greening in SFB cookies was measured at two levels (0.5% and 1%) of cysteine and glutathione. These thiol levels were chosen based on previous work in our group that suggested that lower concentrations of thiols did not prevent CGA-induced greening in SFB cookies (Liang, 2019). Cysteine-containing cookies had diminished greening both immediately after baking and over time (Table 2, Fig. 1), as values for CIE a^* , a measure of greening were consistently higher (less green) in cysteine-treated cookies compared to untreated cookies. Even 24 h after baking, the cookies with 1.0% cysteine were less green than the control cookies, suggesting that 1.0% cysteine's effect was maintained over extended durations. For both ANCOVA analyses describing Cys treated cookies, pH showed a significant effect as a covariate; however, Cys treatment level was not significant (Table 2 and Supplementary Material Table S2). The pH values recorded for cookies with cysteine ranged from 7.97 to 8.75 and decreased by approximately 0.8 pH units at each time period when cysteine concentration was increased to 1.0%. The decrease in pH caused by cysteine likely influences the degree of greening since similar changes in color across small pH differences as

observed in cookies made with xylitol and maple syrup over 24 h were reported by Liang and Were (2018). Their work suggested that a significant increase in internal greening was observed for sunflower cookies when the pH was increased by about 0.2 pH units.

Although Cys lowers greening in the cookies, SFB cookies were still visibly green when formulated with cysteine. The a^* values for cysteine-treated cookies are negative, and the greening increased over time. These observations suggest that cysteine is ineffective in preventing CGA quinone-induced greening at the 0.5% and 1.0% concentrations tested. Increasing cysteine concentrations may diminish greening, particularly over more extended periods. However, high cysteine levels may adversely affect the quality of cookies by altering dough characteristics or generating sulfurous off-notes (Majcher & Jeleń, 2007).

Glutathione did not lower greening at any concentration or time point (Table 2 and Supplementary Material Table S3, Fig. 1), potentially due to the lower molarity of glutathione when compared to cysteine since formulations were based on mass percentages and not molarity. In addition, the pKa of glutathione's relevant ionizable groups, the amine, and the thiol, are more basic compared to cysteine's thiol (2.1, 3.5, 8.7, and 9.1 for the two carboxylic acid, amine and thiol functional groups of glutathione, respectively compared to 1.7 and 8.3 for Cysteine's carboxylic acid and sulfhydryl group). This was reflected in the lack of change in pH of the glutathione containing cookies compared to the corresponding controls which did not contain additional glutathione (Table 2, Supplementary Material Fig. S2). The pH of cookies containing cysteine compared to its controls (Supplementary Material Fig. S2, Table 2 column 4) varied by 0.72, 0.66, and 0.77 pH units after 1, 3, and 24 h respectively, whereas the differences in pH for cookies containing glutathione compared to its controls (Supplementary Material Fig. S2, Table 2 column 8) varied by 0.31, 0.01, and 0.31 pH units after 1, 3, and 24 h respectively.

It was hypothesized that Cys may lower greening by forming conjugates with CGA. The CGA concentrations in cookies formulated with cysteine were 0.41 ± 0.02 – 0.42 ± 0.03 mg CGA/g compared to glutathione-treated cookies with 0.63 ± 0.01 – 0.73 ± 0.01 mg CGA/g. The CGA concentrations were not significantly different from levels quantified in their corresponding controls (cysteine control: 0.42 ± 0.01 ; glutathione control: 0.73 ± 0.01 mg CGA/g), indicating that no or little CGA was depleted by conjugation reactions with protein or added thiols (Fig. 2A and supplementary material S1). In addition, no new peaks that may indicate conjugates were detected (Fig. 2A). Thus, quantifying phenolic compounds in both cysteine- and glutathione-containing cookies provided no direct evidence of conjugate

Table 2
Effect of time and thiol concentration on water activity and pH on CIE a^* values and browning index of sunflower butter cookies.

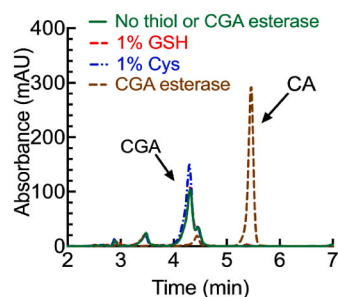
Time [hr]	Thiol Conc [%]	Cookie formulation with cysteine				Cookie formulation with glutathione			
		Water activity	pH	CIE a^*	Browning Index	Water activity	pH	CIE a^*	Browning Index
1	0.0	0.78 ± 0.03^a	8.70 ± 0.13^a	-4.12 ± 1.37^{ab}	45.14 ± 1.41^a	0.78 ± 0.01^a	8.68 ± 0.18^{ab}	-4.98 ± 2.48^a	42.23 ± 4.74^a
	0.5	0.82 ± 0.02^a	8.35 ± 0.16^{ab}	-0.31 ± 0.74^a	50.19 ± 1.85^a	0.71 ± 0.06^{ab}	8.71 ± 0.05^{ab}	-3.27 ± 1.67^a	43.91 ± 0.70^a
	1.0	0.76 ± 0.05^a	7.98 ± 0.12^b	1.39 ± 0.37^a	45.89 ± 0.31^a	0.68 ± 0.03^b	8.37 ± 0.13^b	-2.62 ± 1.96^a	45.42 ± 1.48^a
3	0.0	0.81 ± 0.04^a	8.75 ± 0.11^a	-9.78 ± 1.87^b	29.05 ± 4.31^{ab}	0.75 ± 0.00^{ab}	8.50 ± 0.15^{ab}	-7.27 ± 1.20^{abc}	37.03 ± 2.81^a
	0.5	0.82 ± 0.00^a	8.42 ± 0.06^{ab}	-3.34 ± 1.03^{ab}	42.64 ± 0.70^a	0.69 ± 0.00^{ab}	8.67 ± 0.13^{ab}	-5.86 ± 0.98^{ab}	40.56 ± 1.88^a
	1.0	0.80 ± 0.04^a	8.09 ± 0.11^b	-3.39 ± 1.23^{ab}	42.58 ± 2.12^a	0.67 ± 0.00^b	8.49 ± 0.09^{ab}	-4.39 ± 2.16^a	42.88 ± 2.40^a
24	0.0	0.80 ± 0.01^a	8.75 ± 0.24^a	-11.25 ± 3.05^b	3.11 ± 8.27^{bc}	0.70 ± 0.03^{ab}	9.03 ± 0.30^a	-13.50 ± 0.21^{cd}	15.71 ± 5.66^b
	0.5	0.78 ± 0.01^a	8.25 ± 0.13^{ab}	-10.75 ± 3.11^b	16.68 ± 12.20^{bc}	0.69 ± 0.00^{ab}	8.79 ± 0.04^{ab}	-13.90 ± 1.58^d	12.52 ± 4.15^b
	1.0	0.79 ± 0.00^a	7.98 ± 0.01^b	-5.39 ± 2.92^{ab}	34.56 ± 3.95^{ab}	0.66 ± 0.02^b	8.71 ± 0.18^{ab}	-11.97 ± 1.26^{bcd}	18.96 ± 5.04^b

Values are reported as mean \pm standard deviation of two different formulations for the same treatment. Differences in means were determined by Tukey's HSD test. Values with the same superscripts within the same column are not significantly different at $P > 0.05$.



Fig. 1. Internal greening and browning of cookies formulated and baked with sunflower butter and varying concentrations of cysteine and glutathione.

(A) Phenolic changes in cookies with sunflower butter



(B) Phenolic changes in cookies with sunflower flour

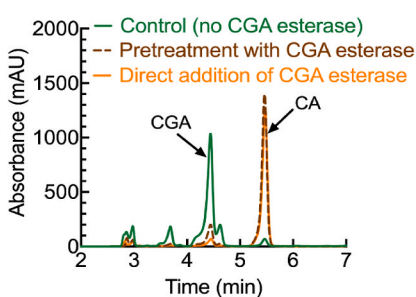


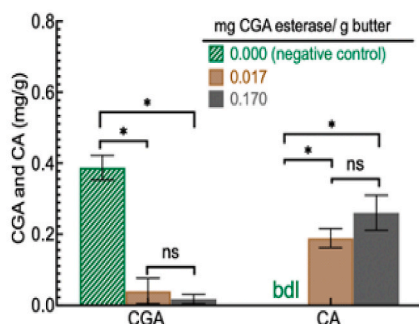
Fig. 2. HPLC chromatograms at 320 nm of (A) untreated sunflower butter cookies (green, solidline), 1% glutathione/GSH (red, dottedline), 1% Cysteine (blue, dashed and dottedline), and 0.17 mg of chlorogenic acid/CGA esterase per g of butter (brown, dashedline). (B) Sunflower flour cookies that received no CGA esterase (green, solidline), were pretreated with CGA esterase (brown, dashedline), or directly treated with CGA esterase (orange, solidline). The chromatograms for each sample are representative of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

formation. However, LC-MS, and SDS-PAGE analysis would be needed to confirm whether small amounts of thioyl-conjugate formation occurred.

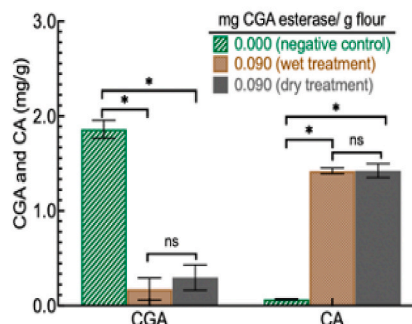
3.3. Chlorogenic acid esterase activity in cookies made with sunflower butter

Our previous work indicated that *Lactobacillus helveticus* CGA esterase cleaves CGA in suspended sunflower meal and prevents

(A) Sunflower butter cookies ± CGA esterase



(B) Sunflower flour cookies ± CGA esterase



* indicates $p < 0.05$ and ns indicates no significant difference

Fig. 3. Chlorogenic acid (CGA) and caffeic acid (CA) level in cookies where (A) CGA esterase was added to sunflower butter and (B) CGA esterase was added to sunflower flour before lyophilization (pretreatment) and after lyophilization (direct addition). Data was analyzed using ANOVA. Error bars show the standard deviation from the mean of two independent sets of dough formulations. * indicates a significant difference at $P < 0.05$ and bdl = below detection limit.

greening in sunflower protein isolates (Lo Verde et al., 2022). Here, we tested whether CGA esterase is active in cookie dough made with SFB, which represents a more complex matrix. The enzyme was added directly to SFB, mixed with the other ingredients, and the dough was incubated for 10 min. CGA esterase was tested at two levels: 1.72×10^{-1} mg/g and 1.72×10^{-2} mg/g CGA esterase per gram of butter. These quantities were chosen based on the kinetic characterization of the enzyme and were expected to result in full cleavage of all CGA in 1 min and 10 min, respectively (Lo Verde et al., 2022). Fig. 2A shows a significant decrease in the peak associated with CGA (retention time/RT = 4.6 min) and a corresponding increase in the peak associated with caffeic acid (RT = 5.5 min) at both enzyme levels. Both levels decreased CGA concentration equally by about 90–95% (Fig. 3A) and resulted in a concomitant increase in caffeic acid. The effectiveness of CGA esterase in SFB is notable since it suggests that the enzyme can be applied directly to the butter and is active in a lipid-rich dough. The high lipid content in the cookie dough does not appear to noticeably impact the activity of CGA esterase, mirroring the results of CGA esterase activity that were documented in undefatted sunflower seed meal (Lo Verde et al., 2022). We note that CGA hydrolysis was exclusively caused by the addition of CGA esterase. Although other cookie dough ingredients such as the commercial flour and eggs may contain hydrolases, control experiments in which no CGA esterase was added showed no CGA removal (Fig. 2A), indicating that any potential hydrolases that may be contained in the other ingredients (flour, eggs etc.) do not act on CGA.

3.4. Chlorogenic acid esterase activity in cookies made with sunflower flour

This study further investigated how CGA esterase prevented greening in cookies made with SFF. Chlorogenic acid hydrolysis was carried out by either pretreating the flour or by directly adding the enzyme to the dough. For pretreatment, SFF was suspended in a buffered solution containing CGA esterase, and after 10 min enzymatic treatment, the meal was lyophilized. The amount of enzyme for both pre- and direct-treatment was 0.09 mg/g of flour, which was expected to result in complete CGA hydrolysis after 2 min (Lo Verde et al., 2022). Enzyme addition resulted in nearly complete elimination of CGA, as shown in Figs. 2B and 3B. CGA hydrolysis was accompanied by CA formation,

further supporting the conclusion that CGA was hydrolyzed (Figs. 2B and 3B). Effective CGA hydrolysis in SFF for both direct and pretreated samples suggests that the enzyme does not need to operate in an aqueous environment. This finding is significant since it indicates that pretreatment of the flour, a process that involved a lengthy lyophilization step, is not necessary and that the enzyme can operate in non-aqueous environments such as cookie dough.

3.5. CGA esterase effect on greening in sunflower flour and butter cookies

Cookies were baked with both treated SFB and SFF. Chlorogenic acid cleavage in esterase-treated cookies was strongly associated with decreased greening. As shown in Fig. 4, Tables 3 and 4, and Supplementary Material Tables S4 and S5, no greening occurred in either SFB or SFF cookies over the entire 24 h monitoring period that received CGA esterase. For both SFB and SFF cookies, greening was lower (higher a^*) and did not change significantly over time in CGA esterase-containing cookies compared to untreated samples after controlling for the effects of pH and water activity. These data indicate that CGA hydrolysis-based greening prevention was successful.

While enzymatic greening prevention worked in both SFF and SFB cookies, the effects on cookie greening and browning are different. The browning index increased substantially in SFB cookies (Table 3). Overall, in SFB cookies, the greenness of CGA-generated trihydroxy benzoic acid compounds appears suitably mitigated (Table 3), however, excessive browning may still represent an aesthetic problem. Browning may arise from the hydrolysis product caffeic acid, which could autoxidize or be involved in Maillard reactions with proteins under alkaline conditions (Namiki et al., 2001; Yabuta et al., 2001).

In cookies made with SFF, greening in untreated control cookies developed more slowly (Table 4). Initially, control cookies were not fully green. However, untreated cookies turned green over 24 h, with a^* values declining from 4.8 to -0.07 . The lack of initial greenness is likely due to the more neutral pH inherent to these (pH = 6.18–7.66), which is maintained over time. CGA esterase-treated cookies do not green at either enzyme application level as CIE a^* values remain constant and positive over time (Table 4). Greening prevention occurs for both cookies that were made with pretreated and directly treated flour, reinforcing the conclusion that pretreatment is not necessary. Browning

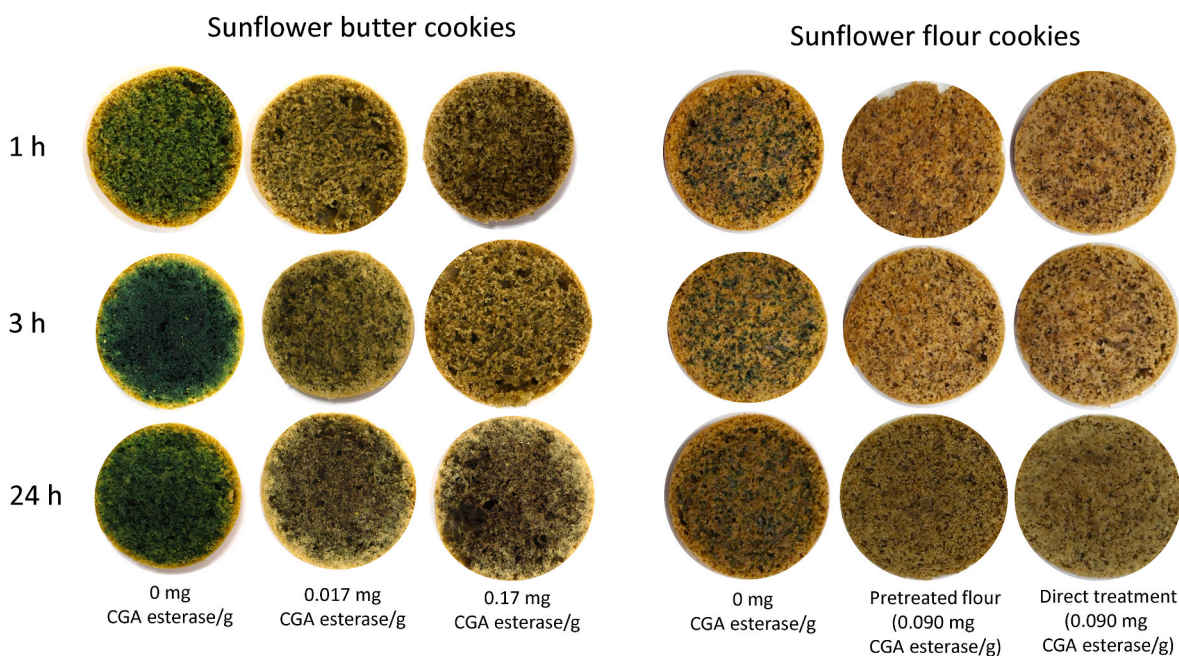


Fig. 4. Internal greening and browning of cookies formulated with sunflower butter and sunflower flour containing varying concentrations and applications of chlorogenic acid (CGA) esterase.

Table 3

Effect of time and esterase concentration on water activity, pH, CIE a^* values, and browning index of sunflower butter cookies.

Time [hr]	Esterase conc [$\times 10^{-2}$ mg/g]	Water activity	pH	CIE a^*	Browning Index	*FRC [$\mu\text{g/g}$]
1	0	0.85 \pm 0.01a	8.91 \pm 0.15ab	-10.38 \pm 1.24c	27.06 \pm 4.60bc	65.13 \pm 10.41a
		0.81 \pm 0.01ab	8.84 \pm 0.01ab	3.29 \pm 0.62ab	49.42 \pm 3.71ab	62.62 \pm 0.51a
	1.72	0.79 \pm 0.02b	8.78 \pm 0.06b	4.44 \pm 0.08ab	46.31 \pm 3.33a	59.70 \pm 8.73a
3	0	0.80 \pm 0.02ab	8.98 \pm 0.02ab	-13.09 \pm 1.15d	12.20 \pm 0.02c	76.66 \pm 8.10a
		0.81 \pm 0.00ab	8.86 \pm 0.00ab	1.88 \pm 0.30b	45.30 \pm 1.00ab	67.02 \pm 19.59a
	17.2	0.80 \pm 0.01ab	8.87 \pm 0.02ab	3.07 \pm 0.76ab	42.52 \pm 1.15ab	57.48 \pm 0.58a
24	0	0.82 \pm 0.03ab	9.07 \pm 0.09a	-11.94 \pm 0.25cd	-10.23 \pm 1.24d	66.40 \pm 21.64a
		0.79 \pm 0.02b	9.00 \pm 0.01ab	3.76 \pm 0.05ab	41.92 \pm 0.71ab	41.02 \pm 0.44a
	17.2	0.79 \pm 0.01ab	8.94 \pm 0.03ab	4.52 \pm 0.03a	44.46 \pm 2.05ab	39.49 \pm 2.32a

Values are reported as mean \pm standard deviation of two different formulations for the same treatment. Differences in means were determined by Tukey's HSD. Values with the same superscripts within the same column are not significantly different at $P > 0.05$.

**Means for Folin-Ciocalteu's reducing (FRC) capacity are included in this table since they are discussed in the main text but are not included in the statistical model for the ANCOVA since FRC is unrelated to cookie color.

was higher in enzymatically treated SFF cookies than in untreated cookies (Table 4). However, compared to cookies made with SFB, SFF cookies were lighter, and the color was more appealing. The SFF cookies formulated with CGA esterase were also much lighter than muffins that were made by Grasso, Liu, and Methven (2020) with baking powder and 15% or 30% sunflower flour. Baking powder does not alkalize the dough, unlike the baking soda used in this study. Furthermore, it is noteworthy that the negative SFF control cookies were beginning to turn green over time despite being at neutral pH, suggesting that maintaining neutral or slightly acidic pH, in and of itself, is not sufficient to produce lightly colored cookies. In summary, the results presented in this section indicate that CGA esterase treatment represents a more effective method of greening prevention than pH modulation.

3.6. Folin Ciocalteu reducing capacity in CGA esterase treated cookies

One of the key benefits of CGA esterase-based greening prevention of SFB and SFF over phenol extraction is that it maintains beneficial antioxidant phenolics. Tables 3 and 4 demonstrate that the redox capacity of cookies, as measured by the Folin reducing capacity, is indistinguishable between treated and untreated cookies. These data confirm that hydrolysis-based greening mitigation does not change the overall Folin Ciocalteu reducing capacity in cookies as the liberated caffeic acid is a

Table 4

Effect of CGA esterase treatment, water activity, and pH on CIE a^* values and browning index of sunflower flour cookies. Folin-Ciocalteu's reducing capacity for each sample is also listed but was not included in the statistical model.

Time [hr]	Flour Treatment	Water activity	pH	CIE a^*	Browning Index	**FRC [$\mu\text{g/g}$]	
1	Control	0.69 \pm 0.04a	6.99 \pm 0.13b	4.80 \pm 1.29b	51.09 \pm 5.31ab	145.23 \pm 17.37abc	
		0.69 \pm 0.01a	6.33 \pm 0.21c	8.95 \pm 0.41a	54.10 \pm 4.99ab	154.57 \pm 7.09abc	
	Direct Treatment	0.69 \pm 0.01a	6.26 \pm 0.10c	9.70 \pm 0.05a	60.09 \pm 1.32a	156.40 \pm 11.10abc	
		0.68 \pm 0.04a	6.45 \pm 0.22c	5.16 \pm 1.31b	44.12 \pm 0.08bc	115.80 \pm 21.48c	
	3	Control	0.65 \pm 0.01a	6.30 \pm 0.10c	8.66 \pm 0.83a	52.26 \pm 2.52ab	168.30 \pm 2.40a
			0.65 \pm 0.03a	6.34 \pm 0.03c	9.69 \pm 0.23a	59.78 \pm 2.22a	141.64 \pm 1.06abc
24	Control	0.69 \pm 0.02a	7.62 \pm 0.06a	-0.07 \pm 0.69c	35.77 \pm 2.30c	117.35 \pm 18.66bc	
		0.68 \pm 0.01a	7.28 \pm 0.06a	8.00 \pm 0.56ab	50.58 \pm 0.86ab	164.82 \pm 5.92ab	
	Direct Treatment	0.67 \pm 0.02a	7.49 \pm 0.04ab	9.84 \pm 1.25a	58.12 \pm 1.58a	152.67 \pm 4.90abc	
		0.67 \pm 0.02a	7.49 \pm 0.04ab	9.84 \pm 1.25a	58.12 \pm 1.58a	152.67 \pm 4.90abc	

Values are reported as mean \pm standard deviation of two different formulations for the same treatment. Differences in means were determined by Tukey's HSD. Values with the same superscripts within the same column are not significantly different at $P > 0.05$.

**Means for Folin-Ciocalteu's reducing (FRC) capacity are included in this table since they are discussed in the main text but are not included in the statistical model for the ANCOVA since FRC is unrelated to cookie color.

potent antioxidant, too.

3.7. Performance of CGA esterase activity compared to commercial enzymes

The activity of CGA esterase is consistent with our earlier work that demonstrated that CGA esterase was active in relatively crowded and dense sunflower meal suspensions, albeit with a lower V_{\max} compared to dilute solutions (Lo Verde et al., 2022). The amount of enzyme used in either SFF or SFB was low. In flour, 0.09 mg/g (90 ppm) of CGA esterase was used, whereas the lowest concentration applied to SFB was 0.0172 mg/g (17.2 ppm). These concentrations are comparable to or well below those of commercially used hydrolases used in the baking industry, such as lipases (Olsen, Si, & Donelyan, 2000; Sato, Sato, & Nagashima, 1991), amylases (Kim & Yoo, 2020) and xylanases (Maat & Martinus, 1990). This suggests that the use of *L. helveticus* CGA esterase is a feasible and efficient method of hydrolyzing CGA in sunflower butter and flour to prevent greening. Utilizing CGA esterase would be compatible with clean label requirements as enzymes are widely accepted and generally recognized as safe for use in food processing (Dai & Tyl, 2021).

4. Limitations of study

The results for cysteine-based greening prevention in sunflower butter cookies were substantially different from those reported for alkaline sunflower protein isolation, in which both cysteine and glutathione showed significant greening mitigation, producing brown-colored protein isolates (Ishii et al., 2021). The cookies were exposed

to heat in contrast to the protein isolates which were never heated. The disulfide bond formation at high heat during baking to form cystine and oxidized glutathione (GSSG) may have consumed the added thiols before any significant greening mitigation could have occurred since oxidation is accelerated at higher temperatures (Dougherty, Singh, & Krishnan, 2017). Differences between the degreening with isolates compared to cookies could be further explained by the complexity of the cookie matrix. Reactions between thiols and other formulation components may have occurred, including disulfide exchange reactions between cysteinyl thiols and the gluten in the flour (Delcour et al., 2012; Tozatti et al., 2020), and Maillard reactions with sugars thus lowering the degreening effect. The limited mobility of cysteine in the dough may also have limited its interaction with CGA phenols (Lagrain, Brijs, & Delcour, 2006; Metzner, 1985).

To discern between cysteine's pH lowering effect and thiol reactivity towards CGA, we attempted to run experiments at tightly controlled pH by increasing the buffer concentration. The expectation was that by keeping the pH of the control and the cysteine containing dough constant, we could discern if greening prevention is based solely on cysteine's pH lowering effect or if other thiol-related reactions were involved. However, it is not possible to fully control the pH in the cookies (even at high buffer concentrations) because of cysteine's own action as a buffer, making these experiments inconclusive.

5. Conclusions

Adding cysteine in dough made with SFB slowed greening in cookies but did not prevent it over time. Cysteine likely acted through its pH lowering effect. In contrast, glutathione was not effective at preventing greening. CGA esterase was very effective at hydrolyzing CGA in both SFB and SFF, fully preventing greening in cookies containing either ingredient. The results from CGA esterase addition to SFB and SFF are, to our knowledge, the first demonstration that SFB and SFF cookies can be made at alkaline pH without green color formation. Unlike existing greening prevention methods, dephenolization is not required, meaning that beneficial phenolic compounds remain in the SFB and SFF. Enzymatic CGA hydrolysis in SFF and SFB may enable more widespread use of sunflower-derived ingredients in high pH baking processes. Greater utilization of SFF would represent a valuable method of "upcycling" abundant sunflower meal produced during sunflower seed pressing and currently discarded or used as animal fodder. Other applications beyond cookie processing, such as bread and muffins, would need to be considered along with sensory evaluation and consumer acceptance studies.

Funding

This work was supported by USDA-NIFA Novel Foods, and Innovative Manufacturing Technologies grant 2020-67018-31261 to L.W. Senger and C. P. Owens., National Science Foundation, Division of Chemistry grant 1905399 to C. P. Owens, and a Research Corporation Cottrell Scholar Award to C. P. Owens. N. B. Pepra-Ameyaw received support from the Chapman University Grand Challenges Initiative and C. Lo Verde acknowledges support by the Chapman Center for Undergraduate Excellence.

CRedit authorship contribution statement

Nana Baah Pepra-Ameyaw: Writing, Formal analysis, Investigation. **Christine Lo Verde:** Writing, Formal analysis, Methodology. **Charles T. Drucker:** Investigation, Writing, Formal analysis. **Cedric P. Owens:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing. **Lilian W. Senger:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

Acknowledgments

The authors would like to thank Dr. Criselda Toto for the insightful discussion on the statistical methods and Gregory Goldsmith for his initial review of the manuscript. The authors thank Drs. Chris Kim, Andrew Lyon, and Marco Bisoffi for the use of instrumentation in their labs.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lwt.2022.114392>.

References

- Adeleke, B. S., & Babalola, O. O. (2020). Oilseed crop sunflower (*Helianthus annuus*) as a source of food: Nutritional and health benefits. *Food Sciences and Nutrition*, 8(9), 4666–4684. <https://doi.org/10.1002/fsn3.1783>
- AOAC. (2019). *Official methods of analysis of the association of official analytical chemists: Official methods of analysis of AOAC international* (21st ed.). AOAC.
- Asther, M., Estrada Alvarado, M. I., Haon, M., Navarro, D., Asther, M., Lesage-Meessen, L., et al. (2005). Purification and characterization of a chlorogenic acid hydrolase from *Aspergillus Niger* catalysing the hydrolysis of chlorogenic acid. *Journal of Biotechnology*, 115(1), 47–56. <https://doi.org/10.1016/j.jbiotec.2004.07.009>
- Bel-Rholid, R., Thapa, D., Kraehenbuehl, K., Hansen, C. E., & Fischer, L. (2013). Biotransformation of caffeoyl quinic acids from green coffee extracts by *Lactobacillus johnsonii* NCC 533. *AMB Express*, 3(1), 28. <https://doi.org/10.1186/2191-0855-3-28>
- Benoit, I., Asther, M., Bourne, Y., Navarro, D., Cnaan, S., Lesage-Meessen, L., et al. (2007). Gene overexpression and biochemical characterization of the biotechnologically relevant chlorogenic acid hydrolase from *Aspergillus Niger*. *Applied and Environmental Microbiology*, 73(17), 5624–5632. <https://doi.org/10.1128/AEM.00374-07>
- Bongartz, V., Brandt, L., Gehrman, M. L., Zimmermann, B. F., Schulze-Kaysers, N., & Schieber, A. (2016). Evidence for the formation of benzacridine derivatives in alkaline-treated sunflower meal and model solutions. *Molecules*, 21(1). <https://doi.org/10.3390/molecules21010091>. Article 1.
- CFR184.1271. (2022). CFR - code of federal regulations title 21. <https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=172&showFR=1&ubpartNode=21:3.0.1.1.3.4>
- Dai, Y., & Tyl, C. (2021). A review on mechanistic aspects of individual versus combined uses of enzymes as clean label-friendly dough conditioners in breads. *Journal of Food Science*, 86(5), 1583–1598. <https://doi.org/10.1111/1750-3841.15713>
- De Vos, D., Collins, T., Nerinckx, W., Savvides, S. N., Claeysens, M., Gerday, C., et al. (2006). Oligosaccharide binding in family 8 glycosidases: Crystal structures of active-site mutants of the β -1,4-xylanase pXyl from *Pseudoalteromonas haloplanktis* TAH3a in complex with substrate and product. *Biochemistry*, 45(15), 4797–4807. <https://doi.org/10.1021/bi052193e>
- Delcour, J. A., Joye, I. J., Pareyt, B., Wilderjans, E., Brijs, K., & Lagrain, B. (2012). Wheat gluten functionality as a quality determinant in cereal-based food products. *Annual Review of Food Science and Technology*, 3(1), 469–492. <https://doi.org/10.1146/annurev-food-022811-101303>
- Dougherty, R. J., Singh, J., & Krishnan, V. V. (2017). Kinetics and thermodynamics of oxidation mediated reaction in L-cysteine and its methyl and ethyl esters in dimethyl sulfoxide-d₆ by NMR spectroscopy. *Journal of Molecular Structure*, 1131, 196–200. <https://doi.org/10.1016/j.molstruc.2016.11.038>
- Drucker, C. T., Senger, L. W., & Pacioles, C. T. (2023). Application of the Weibull model to describe the kinetic behaviors of thiol decolorizers in chlorogenic acid-lysine solutions, 111287 *Journal of Food Engineering*, 339. <https://doi.org/10.1016/j.jfoodeng.2022.111287>. ISSN 0260-8774.
- Esteban-Torres, M., Landete, J. M., Reverón, I., Santamaría, L., de las Rivas, B., & Muñoz, R. (2015). A *Lactobacillus plantarum* esterase active on a broad range of phenolic esters. *Applied and Environmental Microbiology*, 81(9), 3235–3242. <https://doi.org/10.1128/AEM.00323-15>
- EU. (2012). Commission regulation (EU) No 231/2012 of 9 march 2012 laying down specifications for food additives listed in annexes II and III to regulation (EC) No 1333/2008 of the European parliament and of the council text with EEA relevance, 083 <https://op.europa.eu/s/xcrd>.

- Frauenlob, J., Scharl, M., D'Amico, S., & Schoenlechner, R. (2018). Effect of different lipases on bread staling in comparison with Diacetyl tartaric ester of monoglycerides (DATEM). *Cereal Chemistry*, 95(3), 367–372. <https://doi.org/10.1002/cche.10047>
- González-Pérez, S., Merck, K. B., Vereijken, J. M., van Koningsveld, G. A., Gruppen, H., & Voragen, A. G. J. (2002). Isolation and characterization of undenatured chlorogenic acid free sunflower (*Helianthus annuus*) proteins. *Journal of Agricultural and Food Chemistry*, 50(6), 1713–1719. <https://doi.org/10.1021/jf011245d>
- Grasso, S., Liu, S., & Methven, L. (2020). Quality of muffins enriched with upcycled defatted sunflower seed flour. *Lebensmittel-Wissenschaft & Technologie*, 119, Article 108893. <https://doi.org/10.1016/j.lwt.2019.108893>
- Handa, C., Goomer, S., & Siddhu, A. (2012). Physicochemical properties and sensory evaluation of fructooligosaccharide enriched cookies. *Journal of Food Science & Technology*, 49(2), 192–199. <https://doi.org/10.1007/s13197-011-0277-4>
- Ishii, A. K., Toto Pacioles, C., & Were, L. (2021). Color and structural modifications of alkaline extracted sunflower protein concentrates and isolates using L-cysteine and glutathione. *Food Research International*, 147, Article 110574. <https://doi.org/10.1016/j.foodres.2021.110574>
- Kim, H.-J., & Yoo, S.-H. (2020). Effects of combined α -Amylase and endo-xylanase treatments on the properties of fresh and frozen doughs and final breads. *Polymers*, 12(6). <https://doi.org/10.3390/polym12061349>. Article 6.
- Kraehenbuehl, K., Page-Zoerkler, N., Mauroux, O., Gartenmann, K., Blank, I., & Bel-Rhliid, R. (2017). Selective enzymatic hydrolysis of chlorogenic acid lactones in a model system and in a coffee extract. Application to reduction of coffee bitterness. *Food Chemistry*, 218, 9–14. <https://doi.org/10.1016/j.foodchem.2016.09.055>
- Lagrain, B., Brijs, K., & Delcour, J. A. (2006). Impact of redox agents on the physicochemistry of wheat gluten proteins during hydrothermal treatment. *Journal of Cereal Science*, 44(1), 49–53. <https://doi.org/10.1016/j.jcs.2006.03.003>
- Laguna, O., Odinet, E., Bisotto, A., Baréa, B., Villeneuve, P., Sigoillot, J.-C., et al. (2019). Release of phenolic acids from sunflower and rapeseed meals using different carboxylic esters hydrolases from *Aspergillus Niger*. *Industrial Crops and Products*, 139, Article 111579. <https://doi.org/10.1016/j.indcrop.2019.111579>
- Lai, K. K., Lorca, G. L., & Gonzalez, C. F. (2009). Biochemical properties of two cinnamoyl esterases purified from a *Lactobacillus johnsonii* strain isolated from stool samples of diabetes-resistant rats. *Applied and Environmental Microbiology*, 75(15), 5018–5024. <https://doi.org/10.1128/AEM.02837-08>
- Liang, Y. (2019). L-cysteine effects on chlorogenic acid quinone-amino acid induced greening and browning: Mechanism and effects on antioxidant capacity. *Food Science (MS) Theses*. <https://doi.org/10.36837/chapman.000092>
- Liang, S., & Were, L. M. (2018). Chlorogenic acid oxidation-induced greening of sunflower butter cookies as a function of different sweeteners and storage conditions. *Food Chemistry*, 241, 135–142. <https://doi.org/10.1016/j.foodchem.2017.08.084>
- Liang, Y., & Were, L. (2020). Cysteine's effects on chlorogenic acid quinone induced greening and browning: Mechanism and effect on antioxidant reducing capacity. *Food Chemistry*, 309, Article 125697. <https://doi.org/10.1016/j.foodchem.2019.125697>
- Lo Verde, C., Pepra-Ameyaw, N. B., Drucker, C. T., Okumura, T. L. S., Lyon, K. A., Muniz, J. C., et al. (2022). A highly active esterase from *Lactobacillus helveticus* hydrolyzes chlorogenic acid in sunflower meal to prevent chlorogenic acid induced greening in sunflower protein isolates. *Food Research International*, 162, Article 111996. <https://doi.org/10.1016/j.foodres.2022.111996>
- Maat, J., & Martinus, R. (1990). Bread improvers (E.U. Patent number EP0396162A1). European patent office. <https://patents.google.com/patent/US5108765A/en>.
- Majcher, M. A., & Jeleń, H. H. (2007). Effect of cysteine and cystine addition on sensory profile and potent odorants of extruded potato snacks. *Journal of Agricultural and Food Chemistry*, 55(14), 5754–5760. <https://doi.org/10.1021/jf0703147>
- Metzner, A. B. (1985). Rheology of suspensions in polymeric liquids. *Journal of Rheology*, 29(6), 739–775. <https://doi.org/10.1122/1.549808>
- Namiki, M., Yabuta, G., Koizumi, Y., & Yano, M. (2001). Development of free radical products during the greening reaction of caffeic acid esters (or chlorogenic acid) and a primary amino compound. *Bioscience, Biotechnology, and Biochemistry*, 65(10), 2131–2136. <https://doi.org/10.1271/bbb.65.2131>
- Nieter, A., Haase-Aschoff, P., Kelle, S., Linke, D., Krings, U., Popper, L., et al. (2015). A chlorogenic acid esterase with a unique substrate specificity from *Ustilago maydis*. *Applied and Environmental Microbiology*, 81(5), 1679–1688. <https://doi.org/10.1128/AEM.02911-14>
- Nieter, A., Kelle, S., Takenberg, M., Linke, D., Bunzel, M., Popper, L., et al. (2016). Heterologous production and characterization of a chlorogenic acid esterase from *Ustilago maydis* with a potential use in baking. *Food Chemistry*, 209, 1–9. <https://doi.org/10.1016/j.foodchem.2016.03.106>
- de Oliveira Filho, J. G., & Egea, M. B. (2021). Sunflower seed byproduct and its fractions for food application: An attempt to improve the sustainability of the oil process. *Journal of Food Science*, 86(5), 1497–1510. <https://doi.org/10.1111/1750-3841.15719>
- Olsen, T., Si, J. Q., & Donelyan, V. (2000). *Use of lipase in baking (U.S. Patent Number U006110508A)*. United States Patent Office. <https://patents.google.com/patent/US6110508A/en>.
- Pickardt, C., Eisner, P., Kammerer, D. R., & Carle, R. (2015). Pilot plant preparation of light-coloured protein isolates from de-oiled sunflower (*Helianthus annuus* L.) press cake by mild-acidic protein extraction and polyphenol adsorption. *Food Hydrocolloids*, 44, 208–219. <https://doi.org/10.1016/j.foodhyd.2014.09.020>
- R, Core Team. (2021). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. URL <https://www.R-project.org/>.
- Sathya, T. A., & Khan, M. (2014). Diversity of glycosyl hydrolase enzymes from metagenome and their application in food industry. *Journal of Food Science*, 79(11), R2149–R2156. <https://doi.org/10.1111/1750-3841.12677>
- Sato, N., Sato, M., & Nagashima, A. (1991). *Bread improver and method of producing bread (E.U. Patent Number 0468731A1)*. European Patent Office. <https://patents.google.com/patent/EP0468731A1/enit>.
- Siebert, M., Berger, R. G., & Nieter, A. (2018). Enzymatic mitigation of 5-O-chlorogenic acid for an improved digestibility of coffee. *Food Chemistry*, 258, 124–128. <https://doi.org/10.1016/j.foodchem.2018.03.061>
- Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, 16(3), 144–158.
- Soare, E., & Chiurciu, L.-A. (2018). Considerations concerning worldwide production and marketing of sunflower seeds. *Scientific Papers Series Management, Economic Engineering in Agriculture and Rural Development*, 18(3), 421–427. <https://managementjournal.usamv.ro/index.php/scientific-papers/1745-considerations-concerning-worldwide-production-and-marketing-of-sunflower-seeds-1745#pucontentCitation53>.
- Song, Y.-R., & Baik, S.-H. (2017). Molecular cloning, purification, and characterization of a novel thermostable cinnamoyl esterase from *Lactobacillus helveticus* KCCM 11223. *Preparative Biochemistry & Biotechnology*, 47(5), 496–504. <https://doi.org/10.1080/10826068.2016.1275011>
- Tozatti, P., Fleitas, M. C., Briggs, C., Hucl, P., Chibbar, R. N., & Nickerson, M. T. (2020). Effect of L-cysteine on the rheology and baking quality of doughs formulated with flour from five contrasting Canada spring wheat cultivars. *Cereal Chemistry*, 97(2), 235–247. <https://doi.org/10.1002/cche.10239>
- Wianowska, D., & Gil, M. (2019). Recent advances in extraction and analysis procedures of natural chlorogenic acids. *Phytochemistry Reviews*, 18(1), 273–302. <https://doi.org/10.1007/s11101-018-9592-y>
- Wildermuth, S. R., Young, E. E., & Were, L. M. (2016). Chlorogenic acid oxidation and its reaction with sunflower proteins to form green-colored complexes. *Comprehensive Reviews in Food Science and Food Safety*, 15(5), 829–843. <https://doi.org/10.1111/1541-4337.12213>
- Yabuta, G., Koizumi, Y., Namiki, K., Hida, M., & Namiki, M. (2001). Structure of green pigment formed by the reaction of caffeic acid esters (or chlorogenic acid) with a primary amino compound. *Bioscience, Biotechnology, and Biochemistry*, 65(10), 2121–2130. <https://doi.org/10.1271/bbb.65.2121>
- Zhang, W., Liu, Y., Hu, M., & Yang, R. (2019). Preparation of high-quality sunflower seed protein with a new chlorogenic acid hydrolase from *Aspergillus Niger*. *Biotechnology Letters*, 41(4), 565–574. <https://doi.org/10.1007/s10529-019-02654-w>
- Zhang, W., Wang, J., Liu, Y., & Leng, J. (2021). Heterologous expression and characterization of a ferulic acid esterase from *Aspergillus aculeatus* with potential use in sunflower seed processing. *Applied Sciences*, 11(10). <https://doi.org/10.3390/app11104453>. Article 10.